The Role of the IGF System in Somatic Cell and Oocyte Development During Early Bovine Follicular Development

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Abstract

The insulin-like growth factor (IGF) family has been identified as having a key role in regulating follicular growth in mammals. IGFs have been shown to act as survival factors that stimulate somatic cell proliferation and differentiation, as well as being involved in the final stages of oocyte maturation. Insulin-like growth factor binding proteins (IGFBPs) regulate the availability of IGFs as they can sequester extracellular IGFs and hence reduce their bioavailability. The aims of this thesis were to use a serum-free culture system to investigate the role of the IGF system during early bovine follicular development. The study focused on investigating the actions and regulation of IGF in three key stages of follicle growth. These key stages were: primordial follicle initiation, the transition from preantral to early antral stages and early antral follicle growth.

To begin with, this study looked at the effect of IGF-I on primordial initiation and preantral follicle growth and oocyte health, in the presence or absence of androgen. Androgens can have a direct action via androgen receptors (AR), but it has also been suggested that androgens may have an indirect action by influencing the actions of IGF-I through its IGF receptors. No effects of IGF-I on primordial follicle initiation or activated follicle growth were evident, but IGF-I that was not regulated by IGFBPs was found to have a negative effect on the oocyte health of follicles after 6 days of culture. Hence, during the early stages of follicle development regulation of the bioavailability of IGF is crucial to maintain the health of the oocyte. AR immunoreactivity was found in the oocyte and granulosa cells of bovine preantral and antral follicles. Supplementation of culture medium with androstenedione, either on its own or in combination with IGF-I, also failed to affect follicle activation and growth. So the detrimental effect of IGF-I on oocyte health was not altered by the presence of androstenedione.

Previous *in vitro* preantral follicle culture studies have demonstrated a positive effect of IGF-I on follicle growth but a negative effect on oocyte size and granulosa differentiation. In the present study a 6 day culture of bovine early antral follicles in

the presence of IGF-I was found to stimulate both follicle proliferation in the early stages of development and differentiation at all developmental stages, as exhibited by oestradiol production. Furthermore, these effects were found to occur in a dose and stage dependent manner. Additionally, oocyte health was improved by the addition of recombinant IGF-I in the more mature antral follicles. These results highlight the importance of follicle developmental stage when deciding the best *in vitro* culture conditions.

It is important to investigate the mechanisms involved in regulating the expression of IGFBPs at different stages of follicle growth, as changes in their expression will affect the bioavailability of IGF to its receptors. IGFBP-2 mRNA and protein were shown to be expressed in the oocyte and granulosa cells, but not theca cells, of early antral follicles. In addition, IGF-I was found to influence the expression of IGFBP-2 in granulosa cells and oocytes of the largest size range of early antral follicles. A decrease in IGFBP-2 immunoreactivity was observed when follicles were cultured in the absence or in a low dose of IGF-I compared to follicles cultured in a high dose of IGF-I. These results suggest that IGF-I may regulate the expression of IGFBP-2 through a negative feedback mechanism, which again is stage dependent.

The biological actions of IGF depend in part on the ability of specific proteases to break down the IGF/IGFBP complex. The secretion of proteases capable of degrading IGFBP-2 by different bovine follicular compartments was identified, and the effect of IGF-I or follicle stimulating hormone (FSH) on modulating IGFBP-2 proteolytic degradation was also investigated. Proteolysis of IGFBP-2 caused by the incubation of IGFBP-2 with oocytes was not detected. However, incubation of IGFBP-2 with granulosa cells did cause a small level of IGFBP-2 degradation. IGF-I and FSH were not found to enhance or inhibit the degradation of IGFBP-2 after incubation with bovine oocytes or granulosa cells.

The use of a serum-free culture system in this study has improved our understanding of how the complex IGF system is regulated throughout bovine follicular development. It has highlighted that the control of IGF availability to a growing

follicle is governed by different levels of regulation, such as the level of expression and degree of proteolysis of IGFBPs, which are follicle stage dependent. A fuller understanding of the mechanisms that control the access and regulation of local growth factors will allow us to move closer to developing an *in vitro* culture system capable of producing large numbers of viable oocytes for use in reproductive technologies.

Publications and Work Presented from this Thesis

Published papers

Thomas, F.H., Walters, K.A., Telfer, E.E. (2003) How to make a good oocyte: an update on *in-vitro* models to study follicle regulation. Human Reproduction Update, 9(6), 541-555.

Walters, K.A., Binnie, J.P., Campbell, B.K., Armstrong, D.G., Telfer, E.E. (2004) The effects of insulin-like growth factor-I (IGF-I) on bovine follicle development and insulin-like growth factor binding protein-2 (IGFBP-2) expression are dose and stage dependent. (Awaiting submission)

Oral presentations

Walters, K.A., Armstrong, D.G., Telfer, E.E. (2003) The effect of insulin-like growth factor on health and morphology of bovine antral follicles *in vitro*. J Reprod. Fertil. Abstr. Series 2003; 30, Abstract No. O47. Presented at the 3rd Joint Meeting of the British Andrology Society, The British Fertility Society and The Society for Reproduction and Fertility, 13-14 July, 2003, Aberdeen, Scotland, UK.

Walters, K.A., Binnie, J.P., Campbell, B.K., Armstrong, D.G., Telfer, E.E. (2004) The effects of IGF-I and follicle developmental stage on the regulation of IGFBP-2 expression in bovine antral follicles *in vitro*. Presented at the National Ovarian Workshop, 19-21 April, 2004, Edinburgh, Scotland, UK.

Poster presentations

Walters, K.A., Armstrong, D.G., Telfer, E.E. (2003) The effect of insulin-like growth factor on health and morphology of bovine antral follicles *in vitro*. Abstract No.22. Presented at the European Society of Human Reproduction and Embryology Conference, 10-12 April, 2003, Frascati, Italy.

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Walters, K.A., Binnie, J.P., Campbell, B.K., Armstrong, D.G., Telfer, E.E. (2004) The effects of IGF-I on follicle development and IGFBP-2 expression in bovine antral follicles *in vitro*. Abstract No.210. Presented at the 37th Annual Meeting of the Society for the Study of Reproduction, 1-4 August, 2004. Vancouver, Canada.

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Contents

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List of Abbreviations

A4	-	androstenedione
AMH	-	anti-mullerian hormone
ANOVA	-	analysis of variance
AR	-	androgen receptor
ATP	-	adenosine triphosphate
bFGF	-	basic fibroblast growth factor
BMP-4	-	bone morphogenic protein-4
BMP-6	-	bone morphogenic protein-6
BMP-15	-	bone morphogenic protein-15 (GDF-9B)
DNA	-	deoxyribonucleic acid
E2	-	oestradiol
ECM	-	extracellular matrix
EGF	-	epidermal growth factor
Figla	-	factor in the germline alpha
FOXO	-	forkhead transcription factors
FSH	-	follicle stimulating hormone
GDF-9	-	growth differentiating factor-9
GDF-9B	-	growth differentiating factor-9B (BMP-15)
GnRH	-	gonadotrophin-releasing hormone
GVBD	-	germinal vesicle breakdown
HR IGF-I	-	human recombinant insulin-like growth factor-I
17-HSD	-	17β-hydroxysteroid dehydrogenase
IGF	-	insulin-like growth factor
IGF-IR	-	insulin-like growth factor type I receptor
IGFBPs	-	insulin-like growth factor binding proteins
IL-1β	-	interleukin-1beta
IL-6	-	interleukin-6
Insr	-	insulin receptor
IU	-	international unit
IVF	-	*in vitro* fertilisation

IVG	-	*in vitro* growth
IVM	-	*in vitro* maturation
KGF	-	keratinocyte growth factor/ fibroblast growth factor-7
KL	-	kit ligand/stem cell factor
LH	-	luteinising hormone
LIF	-	leukaemia inhibitory factor
LR3 IGF-I	-	Long R^3 insulin-like growth factor-I
MIS	-	müllerian inhibitory substance
MPF	-	maturation promoting factor
mRNA	-	messenger ribonucleic acid
NGF	-	nerve growth factor
OCC	-	oocyte cumulus complex
OGC	-	oocyte-granulosa cell complex
PAPP-A	-	pregnancy-associated plasma protein-A
PCNA	-	proliferating cell nuclear antigen
$P450_{arom}$	-	cytochrome P450 aromatase complex
$P450_{c17}$	-	cytochrome P450 17alpha-hydroxylase
$P450_{scc}$	-	cytochrome P450 cholesterol side-chain cleavage
SDS	-	sodium dodecylsulfate
SEM	-	standard error of the mean
TGFα	-	transforming growth factor alpha
TGFβ	-	transforming growth factor beta
TNF-α	-	tumour necrosis factor alpha
VEGF	-	vascular endothelial growth factor

CHAPTER ONE

Introduction

1.1 General introduction

The main function of the female ovary is to support the differentiation and release of a mature oocyte, which can then be successfully fertilised and ensure the survival of the species. In mammalian ovaries the individual follicle consists of an oocyte, surrounded by granulosa cells and an outer layer of theca cells. These follicles must develop through primordial, primary and secondary stages before reaching the preovulatory stage, and subsequently being ovulated (Figure 1.1). The fate of each follicle is dependent on the integration of both extra-ovarian signals, such as gonadotrophins, and intra-follicular factors, such as locally produced growth factors (Webb *et al.* 2003). These signals and factors determine which follicles will go on to be ovulated or undergo atretic degeneration. The pool of oocytes in the mammalian ovary is generally regarded as becoming fixed early in life, and by the time a female has reached puberty she may have less than 20% of the original number of primordial follicles left (Faddy *et al.* 1987). This represents a huge loss of genetic material and has led to the development of technologies to try to utilise or manipulate this large supply of immature oocytes. Recent findings suggest that germline stem cells may exist and continue to develop in more than the handful of species already known to continue to produce germ cells throughout life (Johnson *et al.* 2004; Telfer 2004). However, even if this is the case, we do not know if these germ cells are viable, or whether they form part of the pool of primordial follicles from which growing follicles are selected throughout the life of the female. At present, the mechanisms that regulate the gradual initiation of growth of ovarian follicles from the primordial pool are poorly understood. By improving our knowledge of the signals that initiate follicular growth in mammals, and of the conditions necessary for sustained early follicle development *in vitro*, improvements in all aspects of fertility can be made. The similarity of bovine and primate ovaries makes cattle excellent models for humans, as well as important models in themselves because of their agricultural importance (Fortune *et al.* 1999). The ultimate goal of studies on follicle growth *in vitro* is to develop conditions that will sustain follicular development to the stage where the oocyte is capable of undergoing normal development, maturation and fertilisation.

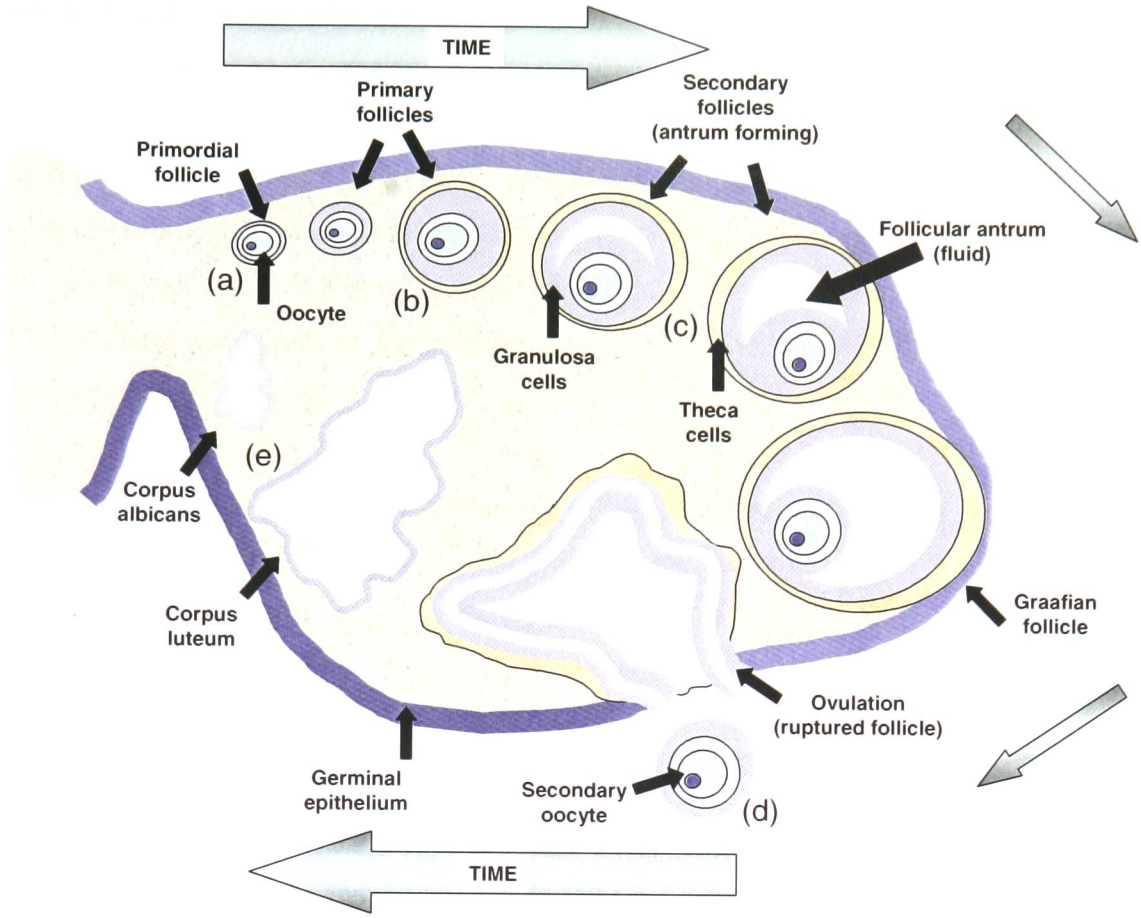


Figure 1.1

Morphology and progression of events during follicular development in mammals

Primordial follicles (a) are embedded in stromal tissue and consist of flattened granulosa cells that enclose a primary oocyte at the dictyate stage with a germinal vesicle (nucleus). After initiation of growth, granulosa cells become cuboidal in shape and begin to proliferate. A theca layer forms and the oocyte grows and secretes the zona pellucida; the follicle is now termed a preantral follicle (b). The production of coalescing fluid drops within the avascular granulosa layer signals the start of antral stages of growth (c). As the follicular antrum fully expands, the oocyte is left surrounded by a distinct layer of granulosa cells known as the cumulus oophorus. Finally, the oocyte is ovulated (d), leaving behind the follicle shell now termed a corpus luteum (e).

The use of assisted reproductive techniques such as superovulation, *in vitro* maturation and fertilisation, and artificial insemination has increased in recent years and had a beneficial impact in improving reproductive performance of many species. However, the safety of reproductive technologies must be fully investigated before being widely applied. In cattle, reproductive efficiency can be described as a

measure of the ability of a cow to become pregnant and produce viable offspring. The average dairy cow in the UK has an optimum calving interval that is dependent to a varying extent on milk yield and season of calving. This financial loss is caused by factors such as loss in milk yield, cost of replacing culled cows, decreased calf sales per cow and an increase in herd depreciation costs. The average calving interval of the UK dairy herd is 395 days, which represents an annual loss of up to £90 per cow, so there is much to be gained from improving the reproductive performance in the dairy herd (Peters and Ball 1995).

Superovulation procedures (which involve bombarding the ovaries with elevated concentrations of gonadotrophins) can increase the number of oocytes, but the response is highly variable, both between and within animals. The numbers of fertilisable oocytes decline with successive treatments, and even when fertilisation is achieved a substantial percentage of the subsequent embryos are lost due to poor oocyte quality prior to conception (Telfer *et al.* 1999). In domestic species there has been only limited success with *in vitro* maturation (IVM) of oocytes recovered from antral follicles by superovulation, possibly due to the follicles being increasingly heterogeneous. The oocytes may therefore already be developmentally compromised. It has been recognised for some time that the best means to increase the capability and success of assisted reproduction programmes is to use oocytes from immature follicles for IVM. Oocytes contained within the hundreds of thousands of primordial and preantral follicles within the ovaries of any individual animal are thought to be a more homogeneous and useful source. The use of new technologies to increase the yield of oocytes and improve female fecundity therefore assumes high priority for research and development.

Although a great deal of research has been focused on methods for growing more developed large preantral follicles *in vitro*, little is known about the conditions necessary to sustain and stimulate the earliest and smallest growing primordial follicles. The ideal culture system would accelerate oocyte/follicle development but still allow key factors to be expressed in the normal sequence without the need for the development of large antral cavities. To fully understand follicle development

and advance technology, it is important for us to identify cellular markers of somatic cell differentiation and oocyte development. These markers need to be able to be related to normal *in vivo* development, and hence signal the health and viability or death of a follicle.

1.2 Follicular formation and follicle growth

The growth of a follicle from the initiation of the primordial stage to the time of ovulation is a dynamic process, which involves a complex system to ensure the oocyte is fully matured and ready for its subsequent fertilisation after it is released from the Graafian follicles at ovulation (Figure 1.2). Follicle growth is characterised by different phases of growth and differentiation, and complex interactions between the gonads, pituitary and the reproductive tract. Each of these components must interact and work together to coordinate the proper sequence of events that lead to oocyte maturation.

1.2.1 Follicular formation

Follicle formation in mammals is the developmental process by which individual oocytes assemble into primordial follicles within the ovary. At the first stage of development, the germ cells stop dividing mitotically and form associations with small numbers of pre-granulosa cells to form primordial follicles (Telfer *et al.* 1988). The germ cell enters meiosis and is now referred to as an oocyte. Meiosis only proceeds to the diplotene stage of prophase before it is arrested (Peters 1969). Primordial follicles constitute a store of germ cells in the postnatal ovary and their numbers vary with species and age, but in the cow and sheep they number between two and three million (Erickson 1966; Gosden and Telfer 1987a; 1987b). The exact timing of follicle formation is species-specific; in rodents, the assembly of follicles is precisely coordinated and occurs during the first few days after birth (Kezele *et al.* 2002a). In contrast, the formation of follicles in domestic animals and primates occurs during fetal life. Furthermore, some follicles begin to leave the resting pool while others are still being formed; hence, follicles are formed over a much longer period of time than in rodents and in a less synchronous manner (Fortune *et al.* 2000; 2003). Factors regulating the crucial process of primordial follicle formation are

largely unknown. However, a gene that has been implicated in regulation of folliculogenesis is Factor in the germline alpha (*Figla*) (Bayne *et al.* 2004; Soyal *et al.* 2000). Knockout analysis in the mouse has shown that *Figla*, a germ-cell specific basic helix-loop-helix transcription factor, is required for oocyte survival and the formation of primordial follicles (Dean 2002; Soyal *et al.* 2000). Additionally, a number of factors have been implicated in the survival of primordial germ cells and the regulation of germ cell proliferation during the crucial period of development leading up to primordial follicle formation. These include activin (Martins da Silva *et al.* 2004), kit ligand and its receptor c-kit (Godin *et al.* 1991; Klinger and De Felici 2002; Manova *et al.* 1993; Robinson *et al.* 2001), bone morphogenetic protein-4 (BMP-4) (Fujiwara *et al.* 2001), Wnt-4 (Vainio *et al.* 1999) and neurotrophins (Anderson *et al.* 2002; Dissen *et al.* 1995; Ojeda *et al.* 2000; Spears *et al.* 2003).

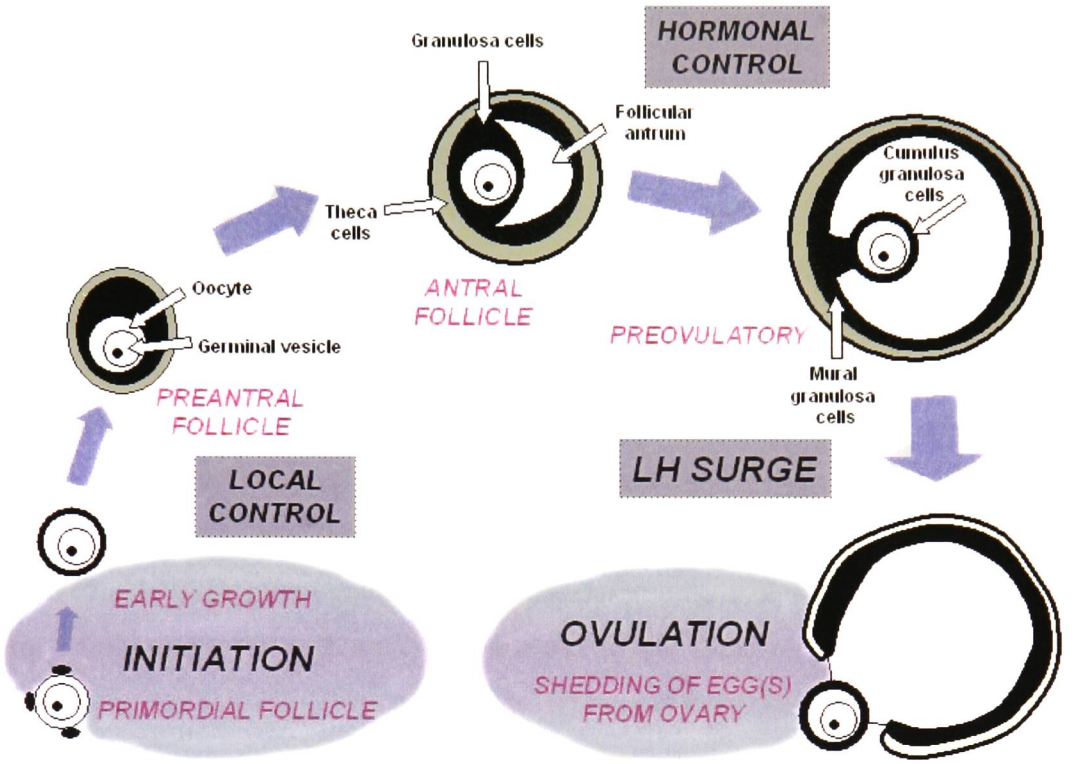
1.2.2 Initiation and early follicle growth

Primordial follicles do not proliferate and therefore represent a female's total reproductive potential, as they are the only source from which groups of ovulable follicles will be recruited for growth throughout life. These follicles remain quiescent until they are triggered by an unknown signal to enter a growing phase. Initially, granulosa cells become cuboidal in shape and begin to express markers of cell proliferation, such as proliferating cell nuclear antigen (PCNA) (Wandji *et al.* 1996; 1997). The oocyte starts to grow (Arendsen and Groen-Klevant 1980; Braw-Tal and Yossefi 1997; Cahill and Mauleon 1981; Lintern-Moore and Moore 1979) and granulosa cells begin to proliferate to form layers around the oocyte. The exact mechanisms responsible for primordial follicle activation, and the mechanisms that permit some of the dormant follicles to initiate growth as soon as the primordial follicle store is established – while the rest of the follicles remain dormant for months or years – remain to be fully elucidated (Picton 2001). Unknown stimulatory factors may be responsible for initiating growth, or alternatively initial recruitment may be due to a release from inhibitory stimuli that maintain the follicles in a resting state (McGee and Hsueh 2000). *Foxo3a* (FKHRL1), a member of the FOXO subfamily of forkhead transcription factors, has been implicated in the regulation of follicle activation. Foxo3a$^{-/-}$ ovaries were shown to contain elevated numbers of early

growing follicles that resulted in the progression of increased numbers of follicles to more advanced stages of follicular development and premature ovarian failure (Castrillon *et al.* 2003). A number of candidate molecules have been suggested for paracrine/autocrine control of primordial development. These include anti-müllerian hormone (AMH) (Durlinger *et al.* 1999; 2002), interactions between c-kit and stem cell factor (kit ligand) (Huang *et al.* 1993; Yoshida *et al.* 1997), and a range of growth factors, including growth differentiating factor-9 (GDF-9) (Dong *et al.* 1996; Elvin *et al.* 1999a; 1999b) and bone morphogenic protein-15 (BMP-15) (Dube *et al.* 1998), but there is no conclusive proof of their involvement. However, gonadotrophins [follicle-stimulating hormone (FSH) and luteinising hormone (LH)] do not appear to be essential for early follicle growth because, in their absence, follicles can still develop to the early antral stage (Awotwi *et al.* 1984; Gong *et al.* 1996).

1.2.3 Preantral to antral follicle development

A preantral follicle is characterised by a primary oocyte, surrounded by a zona pellucida and at least one full layer of cuboidal granulosa cells. During preantral stages the oocyte increases rapidly in volume (Eppig 1994; Schultz and Wassarman 1977), granulosa cells multiply to form several layers, and thecal cells – which are recruited from the surrounding interstitial stromal cells – come to lie on the basement membrane surrounding the granulosa cells (Braw-Tal and Yossefi 1997; Hirshfield 1991b). The early stages of follicle growth are the most crucial for oocyte development as it is during these stages that many of the proteins essential for further development, both before and after ovulation, are produced. The oocyte's diameter increases rapidly during preantral stages of follicle growth due to an increase in protein content (Schultz and Wassarman 1977) and accumulation of resources essential for maturation, fertilisation and pre-implantation embryo development (Eppig 1994). Oocytes from preantral follicles are unable to resume meiosis, but it is during this phase that they are synthesizing molecules essential for the resumption of meiosis (Bachvarova *et al.* 1985).



Follicular dynamics

Follicular stage	Oocyte size (µm)	Follicle size (µm)	Time (days)
Primordial → Preantral	20-60	40-150	>30
Preantral → Early antral	60-100	150-250	15
Antral stages	110-126	250-3670	19
Antral → Pre-ovulatory	126-135	3670→20, 000	8

Follicle/oocyte diameters and period of time spent during each developmental stage

Data adapted from Ginther *et al.* (2001) Webb *et al.* (1999) and Telfer *et al.* (1999).

Figure 1.2

Schematical diagram of the developing follicle and growth rates of bovine oocytes and follicles *in vivo*

1.2.4 Final stages of follicle growth and ovulation

Once the follicle reaches a species-specific size (~200μm for mammalian follicles) granulosa cells start to secrete glycoproteins, which coalesce to form a fluid-filled space called an antrum within the granulosa cell layers (Gosden and Telfer 1987b; Hirshfield 1991a; Lussier *et al.* 1987). This fluid is termed follicular fluid and its composition varies considerably. The formation of the fluid-filled cavity marks the beginning of the antral stage of development. At this stage, the follicles become acutely dependent on gonadotrophins for further growth and development (Nayudu and Osborn 1992). The antral follicle – which precedes the final Graafian follicle (spherical vesicle from which the ovum is released on ovulation) – has three somatic cell compartments that surround the oocyte. These are the differentiated interstitial cells (theca cells), mural granulosa cells and cumulus granulosa cells. The theca cells are vascularised and can be found external to the basal membrane; they express luteinising hormone (LH) receptors. The mural and cumulus granulosa cells are all avascular and express follicle stimulating hormone (FSH) receptors. Mural granulosa cells are located on the inner side of the basal membrane, and as the follicle grows they also begin to express LH-receptors. Cumulus cells are also on the inner side of the basal membrane but separated from the mural cells by the antral cavity. These cells do not express LH-receptors, and at ovulation leave the follicle with the oocyte as a mucified complex. The maintenance of cumulus granulosa cell contact with the oocyte through gap junctions is essential, as the oocyte is dependent on the contact for a supply of nutrients for further development. Granulosa cell contact also holds the oocyte in arrest at the dictyate stage of development until the follicle has reached the appropriate developmental stage, at which point ovulation occurs (Eppig and Downs 1984; Eppig 1991b; Wert and Larsen 1989).

Follicles that have developed to the antral stage may undergo one of two possible fates. One is to continue to increase in size and become a Graafian follicle, which when stimulated by a LH surge will rupture to release the ovum for fertilisation; this process is termed ovulation. The preovulatory follicle undergoes three major changes as ovulation approaches. Firstly, the oocyte resumes meiosis and hence fully matures from a primary to a secondary oocyte. Secondly, there is dissociation of the cumulus

granulosa cells from the granulosa layer; and lastly, the external follicular wall thins and then ruptures (Espey 1967; 1980; Thibault and Levasseur 1988).

However, many antral follicles spontaneously cease to grow and begin to regress or degenerate, a process known as atresia. The mechanism by which follicles are selected for ovulation or atresia is not yet clearly understood. The development of an antrum precedes the occurrence of widespread atresia in the antral follicle population, and as a result of this selection process the vast majority (>99%) of primordial follicles fail to ovulate. This large loss of female germ cells represents a massive wastage of oocytes that could potentially be rescued if developed *in vitro* (Telfer *et al.* 1999).

1.2.5 Oocyte growth and maturation

The limited knowledge on the regulation of oocyte formation, the different steps of folliculogenesis and the required conditions for oocytes to undergo proper growth, differentiation and maturation are major causes of the failure in obtaining viable offspring from *in vitro* cultured early oocytes from domestic species and humans (Van den and Zhao 2005). The growth of the oocyte and accompanying somatic cells from initiation to the final preovulatory stages is a lengthy process, taking around six months in sheep (Cahill and Mauleon 1980) and cattle (Lussier *et al.* 1987). The majority of this time (about four months) is spent in the preantral stages of development, which is the time when the greatest increase in oocyte mass is achieved. During this stage the oocyte enters its extensive growth phase, the surrounding granulosa cells become more proliferative and a theca layer develops around the granulosa from interstitial stroma cells.

For normal fertilisation and embryonic development to proceed, the oocyte must acquire the ability to resume meiosis. Oocytes from immature follicles are unable to resume meiosis (Iwamatsu and Yanagimachi 1975). However, by the time the follicles have reached the antral stages of development the oocytes of most species have acquired the ability to resume meiosis (Mattioli and Barboni 1998; Telfer *et al.* 1999). It is thought the ability of an oocyte to be competent (to be able to resume

nuclear maturation and to undergo fertilisation and cell cleavage) is related to its size. In rodents it was found that oocytes less than 80μm in diameter were unable to complete maturation (Iwamatsu and Yanagimachi 1975). In larger species such as the pig and cow, meiotic competence is acquired in two steps. Oocytes of 100μm in diameter can resume meiosis but do not progress beyond Metaphase I, whereas oocytes of 110μm can progress and complete meiotic maturation to Metaphase II (Fair *et al.* 1995; Hirao *et al.* 1994; Hyttel *et al.* 1997; Motlik and Fulka 1986). Therefore, it appears that the ability of an oocyte to complete meiotic maturation is related to the size of the oocyte and hence the stage of follicle development. Oocytes have to grow to become competent and during this increase in volume, oocytes also have to differentiate. A complex cytoplasmic organisation is required dependent on both the production of new gene products and organelles, and the modification and redistribution of existing ones (Picton *et al.* 2003). Together with a huge rise in RNA and protein synthesis, the numbers of ribosomes, mitochondria and other cell organelles increase enormously in growing oocytes. The accumulation of numerous scattered membrane-bound vesicles, glycogen granules, protein, lipid droplets and multivesicular bodies is indicative for storage and molecular transport across the oocyte membrane. Many organelles transform in appearance and disperse towards the periphery of the oocyte (Van den and Zhao 2005). One of the most crucial changes during the growth phase of an oocyte is its secretion of a glycoprotein membrane, the zona pellucida, which forms a protective coat around the oocyte. The zona proteins are essential for normal follicle development and their expression requires transcription factor Fig-α (Soyal *et al.* 2000). The granulosa cells become coupled with one another and also form gap junctions on the oolemma with the oocyte via processes passing through the developing zona pellucida (Anderson and Albertini 1976). The gap junctions facilitate a two-directional communication and allow transfer of nutrients, metabolic precursors, informational molecules (such as hormones, neurotrophins and growth factors) and inhibitory and stimulatory meiotic signals. By the use of this mechanism the growing oocyte can actively promote growth and differentiation of the somatic cells, while conversely the granulosa cells are able to ensure continued growth and differentiation of the oocyte (Van den and Zhao 2005). During oocyte growth and maturation signalling factors are exchanged

between the oocyte and its surrounding granulosa cells. The oocyte conducts the time of follicular development by controlling granulosa cell proliferation and differentiation, which leads to increased responsiveness to gonadotrophins, theca cell differentiation, cumulus expansion and in the end rupture of the follicular wall (Buccione *et al.* 1990a; 1990b; Erickson and Shimasaki 2000; Salustri *et al.* 1990; Su *et al.* 2002; 2003; Vanderhyden *et al.* 1990; 1992; Vanderhyden and Tonary 1995). In turn, the granulosa cells are indispensable for oocyte growth, differentiation, nuclear meiotic state, cytoplasmic maturation and genomic transcriptional activity (Brower and Schultz 1982; Buccione *et al.* 1990a; Cecconi *et al.* 1991; De La and Eppig 2001; Eppig 1979; Matzuk *et al.* 2002; Tsafriri and Channing 1975; Van den and Zhao 2005).

In early bovine antral follicles, oocytes reach a diameter of 120μm and at this stage acquires competence to resume meiosis, which is associated with almost completely inactivated nuclear transcription activity of the oocyte (Hyttel *et al.* 2001). The competence of an oocyte to resume and complete meiosis and, after fertilisation, to develop into a blastocyst is significantly increased during follicular dominance and particularly during luteolysis through a process called oocyte capacitation, whereby the nuclear envelope becomes undulated and the nucleolar remnant displays a vacuole of increasing size. After termination of oocyte transcription, the oocyte does not enter quiescence but prepares itself for a possible continued development as an embryo after fertilisation (Van den and Zhao 2005). So in growing and dominant follicles, oocytes remain arrested at the diplotene stages of the meiotic prophase, until resumption of meiosis (in fully grown, meiotically competent oocytes from dominant follicles) is triggered by the preovulatory LH surge. During the period between the LH surge and ovulation, the oocyte undergoes a series of marked changes not only in the nucleus but also in the cytoplasm, the process termed as oocyte maturation. Nuclear maturation last about 24 hours in the cow and comprises of several steps (including germinal vesicle breakdown (GVBD) and formation of the first polar body), before the oocyte arrest at MII until fertilisation (Van den and Zhao 2005). Since no LH receptors have been detected in oocytes it is likely that the signal that triggers oocyte maturation originates from their surrounding somatic cells (Peng

et al. 1991). Maturation promoting factor (MPF) is a protein that has been highlight as being responsible for the onset of oocyte maturation as its activation precedes or occurs cocomittantly with GVBD (Gordo *et al.* 2001). *In vitro* maturation of immature oocytes from antral follicles is more rapid than maturing oocyte from earlier stages, but oocyte at this stage are less abundant in mammalian ovaries. Hence, the ability to grow, mature and fertilise oocytes successfully from the most abundant follicle stage in the ovary (primordial follicles) would dramatically increase the reproductive potential of mammalian species.

1.2.6 Follicular growth in cattle

Follicle formation in cattle begins during fetal development and occurs over a long period of time, so that some follicles are activated and leave the resting pool before other follicles have been formed (Fortune *et al.* 2000). Follicular development in cattle is a lengthy process taking around six months from initiation of the growth of primordial follicles until development of a preovulatory follicle (Figure 1.3). The majority of this time (around four months) is spent in the preantral stages of development (Lussier *et al.* 1987).

1.2.6.1 The oestrus cycle

In the non-pregnant cow, ovulation occurs at approximately 21-day intervals. For a short period of time before ovulation, the cow normally exhibits oestrous behaviour or sexual receptivity when she will attract and accept the attention of a bull. Consequently, there is a close relationship between ovarian and behavioural events, ensuring that the female is sexually receptive at the fertile period. The cow is a polyoestrous animal; therefore once oestrous cycles are established they continue indefinitely unless interrupted by pregnancy (Peters and Ball 1995).

Ovarian follicular growth in cattle occurs in waves, usually involving either two or three waves per oestrus cycle (Figure 1.3). These waves are initiated by a rise in circulating FSH for 1-2 days. This rise in FSH triggers the start of each new wave, before gradually declining again (Adams *et al.* 1992; Ginther *et al.* 1997). The follicular waves are first detectable as a group of 4-5mm follicles on approximately

days 0 and 10 for two-wave interovulatory intervals, and on approximately days 0, 9 and 16 for three-wave intervals. Each wave involves the synchronous emergence of a cohort of follicles that begin to grow at a similar rate until one follicle becomes dominant and the remainder become subordinates. The dominant follicle is identified retrospectively as the largest follicle of a wave and may become ovulatory or anovulatory (first wave in two-wave cycles and first two waves in three-wave cycles). Subordinates regress after a short growing phase. The mechanism by which a dominant follicle is selected and continues to grow, while the subordinates regress, is still unknown (Adams *et al.* 1993).

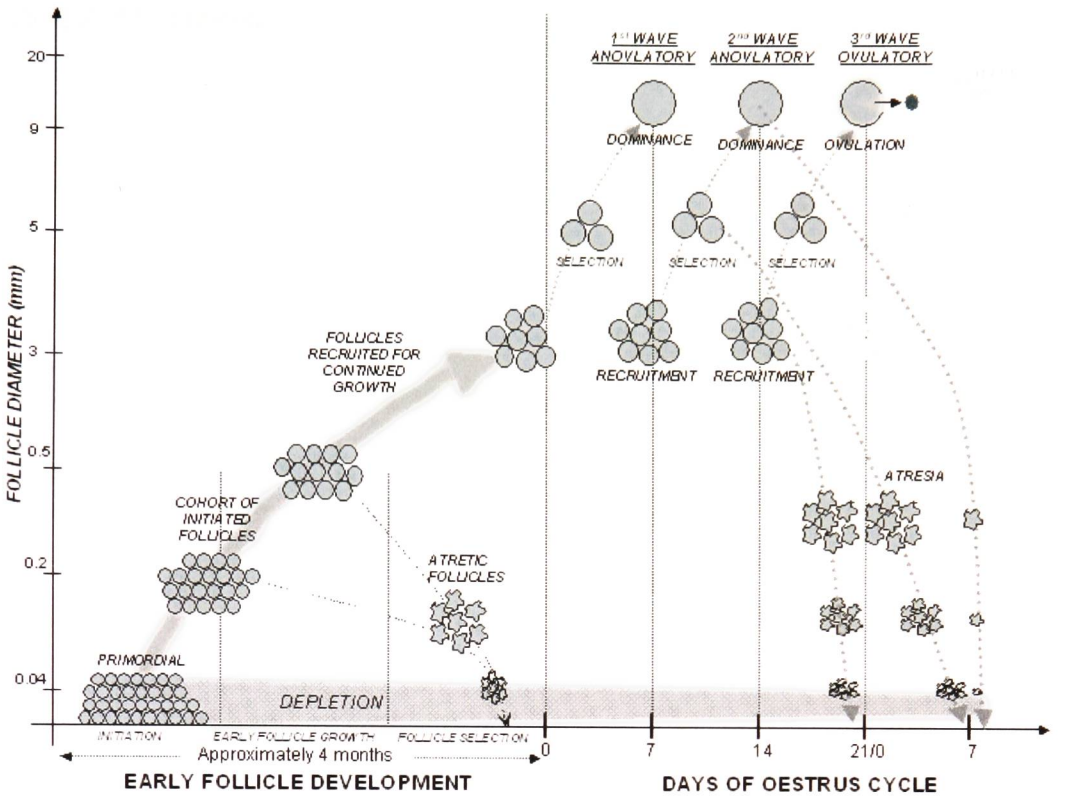


Figure 1.3

Bovine follicular growth and oestrus cycle

Follicular development in cattle is a lengthy process with the majority of this time spent in the preantral stages of development. Cohorts of follicles are triggered to grow; however, most are destined to undergo atresia unless rescued by survival factors. The recruited follicles continue to grow and mature to the antral stage where they need to be capable of responding to gonadotrophins (FSH and LH). Follicles that can respond to gonadotrophins will be selected to develop further and hence escape atresia. Only a few follicles are capable of coping with the decline in FSH levels and subsequently all but one follicle, the dominant follicle, will undergo apoptosis. The dominant follicle goes on to be ovulated, as it is able to maintain a significantly greater level of growth than the subordinate follicles. Two or three waves of follicular development occur sequentially during the bovine oestrus cycle. Following repeated cycles of follicle growth and atresia or ovulation, the follicle reserve becomes exhausted, thus signalling the onset of reproductive senescence.

1.3 Follicle atresia

In mammalian species the vast majority of ovarian follicles, ranging between 70-99.9%, will be eliminated before reaching ovulation (Erickson 1966). Hence, it is the norm for a follicle to die rather than to ovulate. The process by which a follicle is eliminated before reaching ovulation is follicle atresia. Ovarian follicular degeneration or atresia is a hormonally controlled apoptotic process, whereby degenerating follicles are eliminated in a coordinated fashion (Hsueh *et al.* 1994; Kaipia and Hsueh 1997; Markstrom *et al.* 2002).

Follicles die by the process of apoptosis, which is characterised morphologically by detachment of an individual cell from its neighbours, cytoplasmic collapse and cell shrinkage (anoikis), condensation of chromatin and its redistribution to the nuclear perimeter (pyknosis), and the pinching-off (budding) of small spherical bits of plasma (Kaipia and Hsueh 1997; Majno and Joris 1995; Martimbeau and Tilly 1997; Tilly *et al.* 1991; Wyllie *et al.* 1980). Physiological cell death (programmed cell death, apoptosis) differs from that of pathological cell death (necrosis) as it is an active process of gene-directed cellular self-destruction (Tilly *et al.* 1991; Wyllie *et al.* 1980). During apoptotic cell death cleavage of the internucleosomal linker DNA of the nucleus into multiples of 185-200 base pairs occurs under the regulated action of Ca^{2+}/Mg^{2+} dependent endonucleases (Martimbeau and Tilly 1997). Under many conditions, adenosine triphosphate (ATP) generation is needed for the proper execution of physiological cell death, and thus mitochondrial integrity is generally preserved until the final moments preceding cellular dissolution (Martimbeau and Tilly 1997).

The stimuli that trigger the onset of physiological cell death are varied and include both biological (absence or presence of hormones) and pharmacological (irradiation or geotoxicants) agents (Eastman 1993). Gonadotrophins (Braw and Tsafriri 1980; Chun *et al.* 1994; 1996; Kaipia and Hsueh 1997; Markstrom *et al.* 2002), epidermal growth factor (EGF) (Tilly *et al.* 1992), transforming growth factor-α (TGFα) (Tilly *et al.* 1992), basic fibroblast growth factor (bFGF) (Tilly *et al.* 1992), insulin-like growth factor-I (IGF-I) (Chun *et al.* 1994), interleukin-1β (IL-1β) (Chun *et al.* 1995)

and oestrogens (Billig *et al.* 1993) have all been identified as follicle survival factors capable of suppressing apoptotic DNA fragmentation. By contrast, androgens (Billig *et al.* 1993), interleukin-6 (IL-6) (Kaipia and Hsueh 1997), tumour necrosis factor-α (TNF-α) (Kaipia *et al.* 1996), gonadotrophin-releasing hormone (GnRH) (Billig *et al.* 1994) and free radicals (Tilly and Tilly 1995) are potential apoptosis inducing factors.

The regulation of follicular atresia is stage-specific (Markstrom *et al.* 2002). Preantral follicles, although known to be responsive to gonadotrophins, are able to grow to the antral stage without gonadotrophin support (Hirshfield 1991a). However, in culture preantral follicles have been found to undergo spontaneous apoptosis indicating their dependence on survival signals (Kaipia and Hsueh 1997). Therefore, it is likely that intraovarian regulation is of greater importance at this stage of follicle growth. Follicle cell apoptosis occurs at all stages of follicle development, but the antral transition is the 'bottleneck' for developing follicles (Kaipia and Hsueh 1997). At this stage, exposure to FSH seems to be required for their escape from atresia and development to the ovulatory stage, while other factors such as IGF-I and IL-1β have a less pronounced rescuing effect (Hsueh *et al.* 1994; Kaipia and Hsueh 1997).

1.4 *In vivo* studies

In vivo studies using ovariectomy (removal of one or both ovaries) and hypophysectomy (removal of the pituitary gland) techniques have been useful for identifying the function of gonadotrophins (Chiras and Greenwald 1978a; 1978b; Dufour *et al.* 1979; Nakano *et al.* 1975). In addition, the use of ultrasound to track follicles has proved to be helpful in clarifying the changes in follicle diameter, timing and number of waves per cycle (Adams *et al.* 1994; Savio *et al.* 1988; Sirois and Fortune 1988). However, *in vivo* techniques are limiting as they fail to allow individual follicle morphology and health to be analysed; furthermore, it can be hard to see true effects due to the presence of so many factors. Follicular steroidogenesis involves numerous enzymes, and a change in the activity of any one may affect precursor supply to another. In addition, many trophic hormones (hormones influencing the activity of endocrine glands and growth) and paracrine factors

influence steroid synthesis, which in turn further increases the difficulty in deciphering the actions of specific hormones.

1.5 *In vitro* studies

Culture systems provide a well-controlled environment for physiological studies of individual follicles as well as thin cortical strips containing mostly primordial follicles. Systems using serum-free culture medium are preferred as they reduce the numerous undefined components found in serum, which is essential for revealing the function of specific factor(s). These methods allow us to analyse somatic cell and oocyte development and function. They can also provide an insight into how the oocyte interacts with the somatic cells, as these interactions are critical for normal development of the oocyte and follicle unit. Techniques for the culture of ovaries have been described now for almost 70 years. Early *in vitro* techniques used the culture of whole ovaries (Martinovitch 1938), but whilst this type of culture preserves normal tissue interactions, it is severely limited in that long-term maintenance of organ explants is difficult. While it is possible to maintain the cortical regions of the ovary to some extent, the inner medulla region is subject to anoxia, depletion of nutrients and accumulation of metabolites leading to necrosis (Thomas *et al.* 2003b). Due to these limitations and because of the time required for full follicular development (especially in larger species), the culture of adult ovaries is not seen as a viable way to grow and mature oocytes from immature follicles (Eppig and Schroeder 1989; Schroeder and Eppig 1989). Over the past decade, several culture systems have been developed that can produce large numbers of developmentally competent oocytes from immature murine follicles. Eppig and O'Brien (1996; 2003) successfully showed that complete development of mouse oocytes *in vitro* from the primordial follicle stage through to the birth of live young is possible. This amazing achievement depended on a two-step procedure involving organ culture for the follicles to begin growing, followed by the isolation of the growing follicles because organ culture could not sustain full development. By comparison, for domestic animals and humans, the technology is still far from successful due to a variety of reasons including difficulty of follicle isolation, longer period required for follicular growth, and in the case of humans, difficultly in

obtaining a sufficient amount of quality material (containing high numbers of follicles).

1.5.1 Primordial follicle culture

Primordial follicles represent the most abundant population of oocytes in the ovary at any age (Gosden and Telfer 1987a). The isolation of primordial follicles for culture can be achieved by proteolytic enzyme digestion of ovaries (Greenwald and Moor 1989). However, enzymatic techniques lead to degeneration of the basement membrane, which can result in detachment of granulosa cells and denudation of the oocyte (Telfer *et al.* 1999). When follicle integrity is lost, so is the developmental potential of the follicle (Buccione *et al.* 1990a); therefore, an alternative method to obtain primordial follicles has been to culture small strips of fetal ovarian cortex, as these are almost entirely composed of primordial follicles (Braw-Tal and Yossefi 1997; Fortune *et al.* 1998). Wandji *et al.* showed that primordial follicles are capable of initiating in cultured bovine (1996) and baboon (1997) ovarian cortical sections. Furthermore, *in vitro* culture of human primordial and primary follicles within slices of ovarian tissue has shown follicles initiating, developing to secondary follicles and occasionally to early antral follicles (Hovatta *et al.* 1997; 1999; Louhio *et al.* 2000; Wright *et al.* 1999). However, complete development of oocytes from the primordial stage has only been achieved in the mouse (Eppig and O'Brien 1996; O'Brien *et al.* 2003). Initially, this involved organ culture, and once the follicles had begun to grow they were isolated for subsequent follicle culture and *in vitro* oocyte maturation (IVM).

1.5.2 Preantral follicle culture

Several methods for isolating preantral follicles from ovaries (Cortvrindt and Smitz 2001; Smitz and Cortvrindt 2002) have been developed: these include tissue degradation by the use of proteolytic enzymes or collagenase (less damaging to cell membranes) (Eppig and Schroeder 1989; Grob 1964; Roy and Greenwald 1985; 1996; Telfer and Watson 2000; Torrance *et al.* 1989) and manual isolation (Abir *et al.* 1997; McCaffery *et al.* 2000; Nayudu and Osborn 1992; Ralph *et al.* 1995). Enzymatic dissociation procedures can produce high yields of follicles in a shorter

time compared to manual dissection techniques, but manual dissection maintains follicle integrity as an intact theca layer and basement membrane are retained. The viability of rodent culture systems has been demonstrated by the production of live offspring from *in vitro* grown oocyte-granulosa cell complexes (OGC) from mouse preantral follicles (Eppig and Schroeder 1989), the culture of whole preantral follicles (Spears *et al.* 1994), and the two-step procedure of primordial follicle initiation, subsequent follicle isolation and IVM (Eppig and O'Brien 1996; O'Brien *et al.* 2003).

Unfortunately, the isolation and culture techniques from rodent species cannot be readily applied to small bovine follicles due to ovaries from cows being less densely packed with follicles and of a more fibrous nature. They also have a protracted period of follicular growth, and as the follicles increase in size problems of nutrient restriction and insufficient gas and waste exchange arise (Thomas *et al.* 2003b). Furthermore, problems of limited technology, the long generation interval of cattle, and the fundamental lack of knowledge of early stages of follicular development are major obstacles, which have meant that *in vitro* maturation has had little success. However, progress has been made in developing techniques that have so far managed to produce the *in vitro* development of ovine (Cecconi *et al.* 1999; Thomas *et al.* 2002) and bovine (Gutierrez *et al.* 2000; Hulshof *et al.* 1995; Itoh *et al.* 2002; McCaffery *et al.* 2000; Ralph *et al.* 1995; Saha *et al.* 2000) preantral follicles, with some developing to the antral stage. Although no oocytes from bovine or ovine *in vitro* grown preantral follicles have been successfully fertilised, porcine follicles have been shown to develop to the antral stage *in vitro*, produce oocytes capable of resuming meiosis (Hirao *et al.* 1994) and then undergo fertilisation and embryonic development (Wu *et al.* 2001). These studies have established a framework for similar methods to be applied in human and other domestic species. Both mechanical (Abir *et al.* 1997) and enzymatic (Roy and Treacy 1993; Roy and Terada 1999) isolation techniques have been applied to human preantral follicles. Unfortunately, long-term culture of these follicles has met with little success. Late preantral follicles have been maintained in culture for a period of a few weeks, but maintaining a healthy oocyte has proved to be problematic (Abir *et al.* 1997).

1.5.3 Antral follicle culture

Bovine early antral follicles (0.5-0.7mm) cultured for 16 days in serum-free media have been shown to maintain their three-dimensional structures and have morphologically normal oocytes (Senbon and Miyano 2002). However, due to their large size and problems with accumulation of waste products in the culture media, the culture of antral follicles is disappointing. Furthermore, compared to preantral follicles they are much less abundant in number and also have a high possibility of being developmentally compromised at the time of dissection. Therefore, at this stage of development, instead of culturing the whole follicle, work has focused around the removal of the oocyte-granulosa cell complex (OGC) from antral follicles for subsequent oocyte maturation *in vitro*.

1.5.4 Oocyte culture

It is clear that throughout follicular growth the interaction of the oocyte with its somatic cells is fundamental to providing the germ cell with nutrients and growth regulators that are crucial for normal development (Eppig 1991a; 2001; Hunter *et al.* 1976). The ability to mimic the full period of growth of a follicle is an ambitious task, so the creation of a culture environment that supports oocyte-granulosa cell interactions would be beneficial; one such technique is *in vitro* growth (IVG) of immature follicles followed by *in vitro* maturation (IVM) of the oocytes. IVG involves the *in vitro* culture of immature follicles to a time when the oocyte can be collected for subsequent IVM before fertilisation and embryo transfer (Picton 2002; Picton *et al.* 2003). In rodents, IVG/IVM procedures have produced live young (Eppig and Schroeder 1989; Eppig and O'Brien 1996; Roy and Greenwald 1989; Spears *et al.* 1994). However, the use of such techniques in larger mammalian species is problematic due to a longer growth phase, larger follicles and the fact that if the oocytes are recovered from antral follicles for IVM, they are more likely to be developmentally compromised (Telfer *et al.* 2000). Over the past decade many publications using IVM techniques have indicated a variety of factors that could play a role in improving *in vitro* oocyte competence rates (Sirard 2001). These include follicle size (Lonergan *et al.* 1994; Pavlok *et al.* 1992), ovarian stimulation with FSH (Sirard 2001) and culture conditions (Sirard *et al.* 1998); and with a further

understanding of how the follicle induces competence, these conditions and follicular signals can be attempted to be re-created *in vitro*. At present, oocytes aspirated from the antral follicles of domestic species are more commonly used in studies aimed at developing improved culture media and techniques for growing *in vitro* fertilised bovine embryos to the blastocyst stage (Hansel 2003; Lim and Hansel 1996). In humans, some live births have resulted from the maturation and fertilisation of immature human oocytes (Barnes *et al.* 1996; Cha *et al.* 1991; Cha and Chian 1998; Jaroudi *et al.* 1999). In an attempt to improve IVM success rates in the future, methods and techniques for *in vitro* growth (IVG) of immature follicles (primordial and preantral) from human and domestic species are being developed in an attempt to produce large sources of viable oocytes for subsequent IVM procedures (Telfer *et al.* 2000).

1.5.5 Benefits and problems associated with different *in vitro* culture systems

All systems aim to provide an environment which sustains follicular growth. Rodents are excellent models for pioneering technologies as the time period for full follicle growth is short in comparison to domestic species and humans. However, intermediate species are required to test the feasibility for human application. Serum can be added to cultures as it provides a rich environment for *in vitro* follicular development. However, it introduces many unknown elements to the system therefore; serum-free systems are preferred.

Cortical strip cultures allow us to look at the initiation of follicle growth (Braw-Tal and Yossefi 1997; Derrar *et al.* 2000; Fortune *et al.* 1998; Wandji *et al.* 1996), as primordial follicles, which are hard to isolate on there own, have better survival rates when cultured within cortical strips as follicle integrity is not lost (Greenwald and Moor 1989; Telfer *et al.* 1999). To isolate primordial follicles from the adult sheep and cow ovary, a prolonged incubation of several hours is necessary because of the fibrous nature of the ovary. This method leads to a great deal of cellular damage and is therefore not suitable for isolating follicles for culture (Telfer *et al.* 1999). Small isolated of pieces of ovarian cortex have high densities of primordial follicles and are hence a more productive way to culture primordial follicles. This culture system also

allows us to study follicle interactions, as paracrine interactions are maintained however, this system is not well defined as each strip is heterogeneous as it is influenced by follicles at different stages of development present throughout the strip; it is difficult to track an individual follicles growth and can only be used for follicles at early stages of development.

Culture of isolated follicle provides an environment whereby addition of factors can be controlled and their action on follicle growth and health can be studied. Furthermore, particular developmental characteristics can be related to subsequent reproductive performance of the gamete, thereby defining normal follicular development more accurately (Thomas *et al.* 2003b). Individual follicle culture provides a well controlled environment where a single follicle can be tracked. Follicles can be isolated by the use of enzymes (Eppig and Schroeder 1989; Grob 1964; Roy and Greenwald 1985; 1996; Telfer and Watson 2000; Torrance *et al.* 1989) or by mechanical isolation with needles (Abir *et al.* 1997; McCaffery *et al.* 2000; Nayudu and Osborn 1992; Ralph *et al.* 1995). Enzymes allow large numbers of follicle to be isolated in a short period of time but, there are problems with enzymes causing degradation of the basement membrane. Microdissection, on the other hand, maintains the three-dimensional structure of the follicles but is very time consuming (Thomas *et al.* 2003b). Oocyte-granulosa cell complexes (OGC) can be isolated from preantral follicles by the use of enzymes to provide an important tool for the study of oocyte development as well as for analysis of follicular development and function. In mouse follicles, degradation of the theca cell layer and the basement membrane by collagenase results in OGC, and these have been cultured to produce oocytes that have grown and acquired developmental competence (Eppig and Schroeder 1989). This system appears to be the best for producing oocytes but it lacks other components of the follicle such as theca and antral cavity, however, the viability of the rodent system using whole mouse follicles has been demonstrated by the production of live young (Spears *et al.* 1994).

The use of manual dissection to retrieve individual follicles maintains a theca layer and an intact basement membrane therefore, follicle integrity is maintained, which is

important for the examination of interfollicular effects and steroidogenesis. This system maintains the three-dimensional organization of cells, and the interactions of the various follicular cell types which is critical for normal cellular development however, follicle-follicle interactions are lost and in large species (such as cattle and humans) when follicles reach a large size there can be problems of insufficient waste and gas exchange.

The culture of oocyte-cumulus complexes (OCC) from antral follicles allows later stages of oocyte development to be studied (Picton 2002; Fair *et al.* 2002) when *in vitro* culture of the whole follicular unit is prohibited due to problems with accumulation of waste products in the culture media. Large numbers of OCC can be easily and cheaply attained by the aspiration of antral follicles. The *in vitro* culture of OCC allows investigation of factors influencing oocyte maturation and fertilisation, bearing in the mind that the three-dimensional structure of the follicular unit is lost and oocytes aspirated from antral follicles may already be developmentally compromised (Telfer *et al.* 1999).

1.6 Oestrogen biosynthesis

Oestrogens are well-known endocrine and intrafollicular autocrine mitogenic compounds (Tonetta and diZerega 1989). In mammals, these steroids are generally synthesised in granulosa cells of antral follicles, but in sows and women this also occurs in the theca cells (Van den Hurk *et al.* 1997). Oestradiol synergises with FSH to enhance granulosa cell proliferation and antrum formation in large preantral follicles in the rat (Tonetta and diZerega 1989) and pigs (Hirao *et al.* 1994). By contrast, however, oestradiol does not affect the proliferative activity of bovine granulosa cells, but it has been shown to enhance their size in cultured bovine primary and small secondary follicles (Hulshof 1995).

In monovular species, the preovulatory follicle is responsible for inducing oestrus and the ovulatory LH surge, which it does by increasing the secretion of the steroid oestradiol (Silva and Price 2000). Thus the ability for a follicle to synthesise oestradiol is essential for a normal, healthy follicle to develop. The main oestrogens

produced in the ovary are oestradiol 17β and oestrone. Oestradiol is produced by an interaction between the steroidogenic granulosa and theca cells under the regulation of FSH and LH. Oestrogen biosynthesis is achieved by a two-cell, two-gonadotrophin mechanism, with granulosa cells being the major site of conversion of androgens to oestrogens (Hillier *et al.* 1994) (Figure 1.4). To provide the granulosa cells with androstenedione [the preferred substrate in cattle (Silva and Price 2000)] – which is then converted to oestrogen – acetate and cholesterol are converted to androgens within the vascularised theca cells by the rate limiting enzyme cytochrome P450 17alpha-hydroxylase ($P450c_{17}$) (Hillier *et al.* 1994; Sasano *et al.* 1989). This conversion is regulated and greatly stimulated by LH (Erickson *et al.* 1985).

Granulosa cells are incapable of producing androgens. However, when the avascular mural granulosa cells are provided with exogenous androgens produced by the theca cells they readily aromatise them to oestrogens under the stimulation of FSH. This process involves the conversion of androstenedione into oestrone by cytochrome P450 aromatase complex (P450arom) (Simpson *et al.* 1994). Oestrone is then converted to oestradiol by the enzyme 17β-hydroxysteroid dehydrogenase (17βHSD) (Silva and Price 2000).

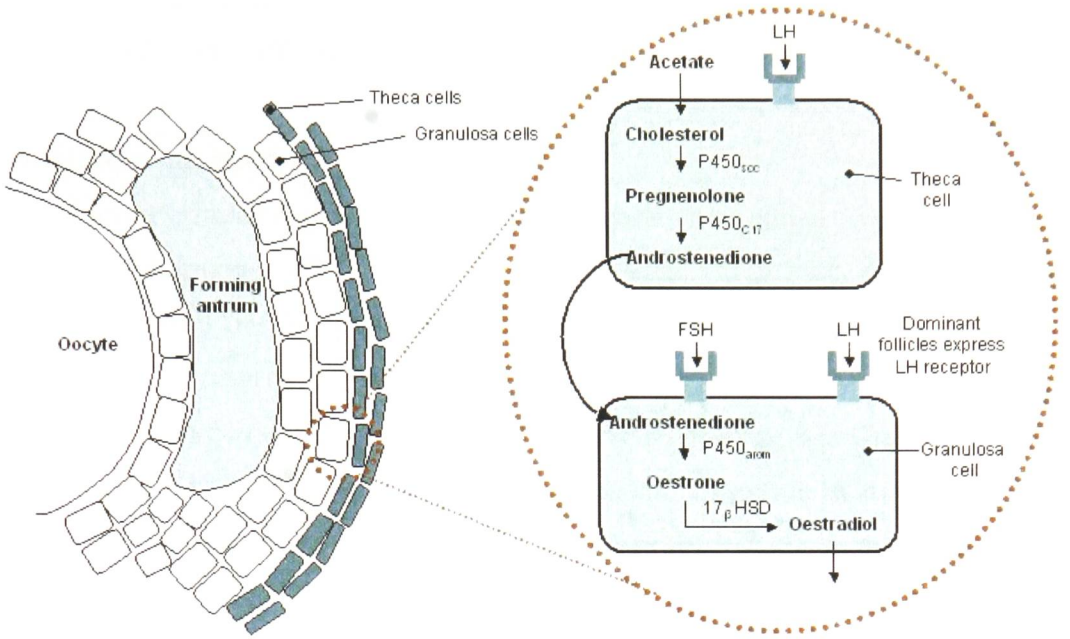


Figure 1.4

The 2-cell, 2-gonadotrphin mechanism for follicular oestrogen biosynthesis

Androgen synthesis occurs in the theca cells and is regulated by LH. Cholesterol is converted into androgens under the regulation of LH. These androgens are then diffused into the granulosa cells, where P450arom aromatises them into oestrogens in response to FSH.

Oestrogens stimulate further oestrogen output by binding to receptors in the granulosa cells, which are then stimulated to proliferate and also to synthesize yet more oestrogen receptors (Hillier *et al.* 1994). Steroidogenesis has been shown to be enhanced by other factors such as insulin and insulin-like growth factor-I (IGF-I) (Bergh *et al.* 1991; Campbell *et al.* 1998; Cara and Rosenfield 1988; Spicer *et al.* 1993; Stewart *et al.* 1995; Zhao *et al.* 1998), either working alone or in synergy with gonadotrophins. The production of oestradiol increases as the follicle develops, which means large antral follicles secrete much greater amounts of oestradiol than small antral follicles (Smith *et al.* 1996). Therefore, measuring the amount of oestradiol being secreted can give a good estimation of the oestrogenic capability of a follicle and can indicate if the follicle is healthy. A decrease in oestradiol synthesis is one of the early signs of follicle atresia (Guilbault *et al.* 1993). Peak follicular

oestradiol concentrations are generally obtained during the growth phase of the dominant follicle, and then oestradiol concentrations decrease considerably as the follicle reaches its maximum diameter (Badinga *et al.* 1992; Price *et al.* 1995).

1.7 Endocrine control

The anterior pituitary gland is known to secrete the protein hormones follicle stimulating hormone (FSH) and luteinising hormone (LH), both of which have a critical influence on the reproductive system. The synthesis and secretion of both FSH and LH is controlled by gonadotrophin releasing hormone (GnRH), which is a member of a large family of peptides and is produced in the hypothalamus (Austin and Short 1972). FSH and LH are involved in endocrine control of follicle development, mainly by regulating the expression of the P450 steroidogenic enzymes necessary for ovarian steroidogenesis (Hillier *et al.* 1994). FSH is the key hormone stimulating the emergence of waves of follicles, and its decline is associated with the selection of a dominant follicle, which then becomes dependent on LH for its final fate when concentrations of FSH are minimal (Roche 1996). The early stages of follicle growth can occur in the absence of gonadotrophins (Dufour *et al.* 1979); however, the later stages of preantral and early antral development are primarily regulated by gonadotrophins. FSH is an essential survival hormone for the prevention of atresia of antral follicles (Chun *et al.* 1996; Hirshfield 1991a; Hsueh *et al.* 1994). It is vital for the formation of the antral cavity (Nayudu and Osborn 1992) in mouse ovarian follicles cultured *in vitro* and is also involved in the proliferation and differentiation of granulosa cells (rodent: Roy and Greenwald 1989; Tonetta and diZerega 1989; cattle: Hulshof *et al.* 1995; Ralph *et al.* 1995; pig: Hirao *et al.* 1994; Morbeck *et al.* 1993; sheep: Newton *et al.* 1999; human: Roy and Treacy 1993) and in the maturation of the oocyte prior to ovulation (Durinzi *et al.* 1997; Eppig and Schroeder 1989; Gilchrist *et al.* 1997; Guler *et al.* 2000; Schramm and Bavister 1994).

Although early follicle development has been shown to occur independently of gonadotrophins (Dufour *et al.* 1979), the presence of FSH binding sites/receptors in granulosa cells of humans (Zheng *et al.* 1996), cattle (Wandji *et al.* 1992), sheep

(Eckery *et al.* 1997) and rats (Monniaux and de Reviers 1989) supports a physiological role for FSH during early stages of follicle growth. Furthermore, recent findings have shown FSH can affect the rate of development of preantral follicles in mice (Mizunuma *et al.* 1999), rats (McGee *et al.* 1997), humans (Wright *et al.* 1999) and sheep (Campbell *et al.* 2004).

1.8 Intraovarian control

Although follicular development is primarily regulated by gonadotrophins, other factors have been shown to alter follicular growth patterns. A range of follicular growth factors are now known to be involved in the regulation of follicular growth. Factors such as basic fibroblast growth factor (bFGF) (Nilsson *et al.* 2001) and nerve growth factor (NGF) (Dissen *et al.* 2001) have been associated with inducing primordial follicle development and activating follicle growth, while anti-műllerian hormone (AMH) inhibits initiation of primordial follicle growth (Durlinger *et al.* 1999; 2002). Leukemia inhibitory factor (LIF) (Nilsson *et al.* 2002) has also been shown to promote the primordial to primary follicle transition, while vascular endothelial growth factor (VEGF) (Danforth *et al.* 2003) and műllerian inhibitory substance (MIS) (McGee *et al.* 2001) have been reported to stimulate preantral follicle growth. Yet another factor implemented in modulating early stages of follicle development is epidermal growth factor (EGF) (Roy 1993; 1999).

The transforming growth factor-β (TGF-β) superfamily comprises a range of proteins, including members of the activin-inhibin system, with the potential to act as intraovarian regulators (Armstrong and Webb 1997). Activin suppresses androgen output by the theca cells but stimulates the granulosa cells to develop aromatising capacity. Inhibin, on the other hand, stimulates androgen output and moderates aromatising capacity. Local feedback loops may function within individual follicles involving a sequential change of inhibins, activins and their binding proteins, which ultimately determines the different fates of the selected and unselected follicles that develop in the same systemic environment of gonadotrophins and growth hormone (Roche 1996). Therefore, the interaction of a complex range of intrafollicular, extra- and intraovarian signals determines the ultimate fate of the follicle. It has become apparent that the insulin-like growth factor (IGF) system plays a central role in these

interactions, regulating both the proliferative and steroidogenic responses of thecal and granulosa cells to gonadotrophins (Armstrong *et al.* 1998).

1.9 Regulation by oocyte-specific factors

It is well known that granulosa cells can regulate oocyte meiosis by direct transfer of meiosis-arresting signals through gap junctions (Tsafriri and Channing 1975). However, evidence is now rapidly accumulating that oocytes themselves can profoundly influence the development and function of a growing follicle. Oocytes are now thought to play an essential role in controlling their own development by regulating the function of oocyte-associated granulosa cells (Buccione *et al.* 1990a; 1990b). Data supporting this view has shown that oocytes promote granulosa cell proliferation (Vanderhyden *et al.* 1990; 1992) and alter steroid production (Vanderhyden and Tonary 1995). Furthermore, the oocyte has been found to be essential in stimulating hyaluronic acid synthesis by the cumulus granulosa cells (Salustri *et al.* 1990), resulting in the morphological process of cumulus expansion (Buccione *et al.* 1990a; 1990b). This stimulation of hyaluronic acid synthesis is brought about by the production of the oocyte-secreted enabling factor, which is thought to be made up of one or more oocyte-secreted paracrine factor(s) (Eppig *et al.* 1993; Vanderhyden *et al.* 1990). The TGF-β superfamily consists of secreted peptide growth factors that are known to be important in a variety of developmental events during ovarian follicle development (Knight and Glister 2003). Three members of the TGF-β superfamily are known to be expressed by oocytes: these are growth differentiation factor-9 (GDF-9), bone morphogenetic protein-15 [BMP-15 (also known as GDF-9B)] and bone morphogenetic protein-6 (BMP-6) (Aaltonen *et al.* 1999; Bodensteiner *et al.* 1999; Elvin *et al.* 2000; Jaatinen *et al.* 1999; McGrath *et al.* 1995; McNatty *et al.* 2001).

Dong *et al.* (1996) uncovered the family member of the TGF-β superfamily called GDF-9. This oocyte-secreted factor has been found to be obligatory for proper follicle growth and fertility in mice. Ovaries from GDF-9-deficient female mice demonstrated that primordial and primary one-layer follicles will form; however, there is a block in follicular development beyond the primary one-layer follicle stage

that leads to complete infertility. GDF-9 is now known to be expressed in rodent, human (McGrath *et al.* 1995) and ruminant (Bodensteiner *et al.* 1999) oocytes, and in the porcine oocyte and cumulus cells (Prochazka *et al.* 2002; 2004). Further *in vitro* studies have so far shown that GDF-9 can promote kit ligand mRNA expression (Nilsson and Skinner 2002) and stimulate rat theca cell androgen biosynthesis (Solovyeva *et al.* 2000), as well as promote the growth, development and survival of human follicles at early stages of growth (Hreinsson *et al.* 2002). Furthermore, the effects of GDF-9 on mice granulosa cells change with the progression of their development (Latham *et al.* 2004).

In contrast to the effects of GDF-9 gene knockout in mice, the deletion of the BMP-15 or -6 gene in mice had little or no effects on follicle development and fertility (Solloway *et al.* 1998). However, a naturally occurring mutation in BMP-15 causes a striking effect on the fertility of the Inverdale and Hanna lines of sheep (Galloway *et al.* 2000).

Yet another factor known to be expressed in murine and ovine oocytes is the tyrosine kinase receptor c-kit, with its ligand (kit ligand) being found in the granulosa cells (Motro and Bernstein 1993; Clark *et al.* 1996; Tisdall *et al.* 1997). Inhibition of the interaction between KL and c-kit prevents the transformation of murine primordial follicles to primary follicles (Huang *et al.* 1993; Yoshida *et al.* 1997). The interaction of this ligand with its receptor also appears to be important after initiation of follicle growth, as oocyte growth was found to accelerate in murine preantral follicles when cultured in the presence of KL (Packer *et al.* 1994). Further to the factors discussed above, oocytes may secrete many proteins during their development whose functions, at the present time, remain unknown (Eppig *et al.* 2002).

1.10 Insulin-like growth factor system

The insulin-like growth factor (IGF) ligands, IGF-I and IGF-II, were first purified and sequenced in 1978 (Rinderknecht and Humbel 1978a; 1978b). Following this purification, extensive research has been carried out on these peptides and the other constituents of the IGF family. The findings of Adashi *et al.* (1985), findings were

published on the role of IGFs as intraovarian regulators of granulosa cell growth and function. Since this journal, the accumulation of information on the involvement of the IGF system in the ovary has increased rapidly. The structures of the components of the IGF family have now been described, and an understanding of the regulation and function of these peptides and proteins in the ovary has begun to emerge.

1.10.1 The insulin-like growth factor family

The IGF autocrine/paracrine system is a complex family made up of the IGF peptides (IGF-I and IGF-II), specific IGF receptors (type-I and type-II) in their target cells, a family of proteins called insulin-like growth factor binding proteins (IGFBP) that regulate the availability of the IGFs to their target cells, and IGFBP proteases (specific and non-specific) (Figure 1.5) (Giudice 1992).

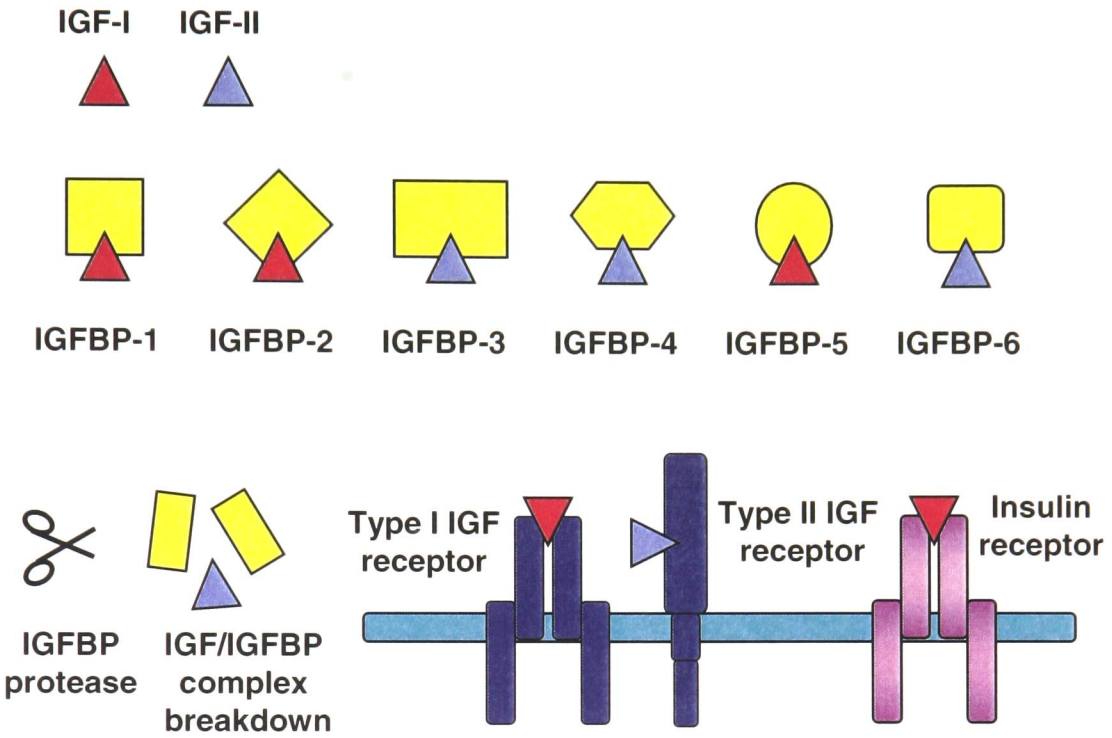


Figure 1.5

The insulin-like growth factor (IGF) system

The IGF system consists of two IGF ligands I and II, insulin-like growth factor binding proteins (IGFBPs) of which there are at least six known, two IGF receptors (type I and type II) and the IGFBP proteases. The ligands bind to the receptors to exert an action on the cell. Their bioavailability can be regulated by their interaction with the IGFBPs; when an IGF is bound to an IGFBP it cannot exert an action. However, the IGF/IGFBP complex can be broken down by the action of proteases that cleave the IGFBP and hence release the IGF. Under certain conditions both IGF ligands can interact with the insulin receptor to mediate their action.

IGF-I and IGF-II, which are structurally similar to insulin, are two highly homologous, low molecular weight hormone peptides that promote cellular mitosis and differentiation (Giudice 1992). The IGFs are single chain polypeptides that contain three intrachain disulphide bonds. The IGFs are known to be important mitogens that affect cell growth and metabolism. As well as endocrine effects

exerted by circulating IGFs, locally produced IGFs exert paracrine, as well as autocrine, effects on cell proliferation (Hammond *et al.* 1991; Hwa *et al.* 1999).

The IGFs (IGF-I and -II) facilitate their actions by binding with their specific cell surface membrane receptors. Both of them can also, under certain conditions, interact with the insulin receptor (Hwa *et al.* 1999) at lower affinity. IGF-I preferentially interacts with IGF-I receptor and the same is also true for IGF-II and the IGF-II receptor. The type I IGF receptor has, in most tissues, a higher affinity for IGF-I compared to IGF-II, and it binds insulin with moderate affinity (Giudice 1992). The type I IGF receptor and the insulin receptor are glycoproteins that are tetramers, consisting of two α-subunits and two β-subunits, which are linked by interchain disulphide bonds (Ullrich *et al.* 1986). The α-subunit is extracellular and contains the ligand-binding domain, whereas the β-subunit has a hydrophobic domain that traverses the cell membrane, contains an ATP binding site and ligand-activated tyrosine kinase activity, as well as sites for auto phosphorylation. The type II IGF receptor has a high affinity for IGF-II, a lower affinity for IGF-I and does not bind insulin. Its structure differs from that of the type I IGF receptor and the insulin receptor in that it is a single chain glycoprotein, with ninety percent of its linear structure being extracellular (Giudice 1992). The Type II IGF receptor primarily binds IGF-II, but also serves as a receptor for mannose-6-phosphate-containing ligands (Hwa *et al.* 1999).

In many biological fluids, such as serum, amniotic fluid and other body fluids, the IGFs are usually found bound to IGFBPs. The IGFBPs constitute a heterogeneous group of at least six distinct proteins (Figure 1.6) capable of binding IGFs with affinities in the 10^{-10} to 10^{-9} M range (Adashi 1998; Hwa *et al.* 1999; Spicer and Echternkamp 1995). IGFBPs are cysteine-rich proteins sharing high similarity in their primary amino acid sequence. They have three recognised domains: the conserved N-terminal domain, the highly variable mid-region, and the conserved C-terminal. The N- and C-terminus have been shown to be necessary for high affinity binding of the IGFs, with the loss of the C-terminus significantly reducing the ability of the IGFBP to interact with IGFs. There are two distinct classes of IGF binding

proteins: these consist of the low-affinity (IGFBP related proteins and IGFBP proteolytic fragments) and the high affinity IGF binders (IGFBPs 1-6) (Hwa *et al.* 1999). A number of factors have been identified in regulating IGFBP serum levels, including nutritional status (Zapf *et al.* 1989), peptide hormones, proteases and chronological age (Donahue *et al.* 1990).

1.10.2 Action of IGF and IGFBPs in the ovary

Components of the IGF system have been identified as being expressed and regulated in ovaries of many mammalian species; this strongly supports a central role for the IGF system in ovarian follicular development. Furthermore, IGFs affect biosynthetic processes in granulosa and theca cells and have an influence on mitotic activity in the granulosa cells (Giudice 1992; Poretsky *et al.* 1999). The IGF system plays a key role in ovarian function: an absolute requirement for IGF-I has been highlighted in experiments using IGF-I knock-out mice that were sterile (Baker *et al.* 1996). *Igf1* null mice have impaired granulosa cell proliferation and follicular growth (Kadakia *et al.* 2001). IGF-I has been shown to have a role in both proliferation and differentiation of cattle (Spicer *et al.* 1993; Stewart *et al.* 1995), sheep (Campbell *et al.* 1998; Monniaux and Pisselet 1992), pig (Kolodziejczyk *et al.* 2003) and human (Bergh *et al.* 1991; Zhao *et al.* 1998) granulosa and theca cells *in vitro.* In the early stages of follicle growth, IGF-I has been found to act as a survival factor (Louhio *et al.* 2000; Wandji *et al.* 1998) and to stimulate follicular growth (Gutierrez *et al.* 2000; Itoh *et al.* 2002). IGF-I is important in the antral stages of follicle development, where it is involved in the regulation of follicle growth, stimulation of somatic cell proliferation and oestrogen, as well as progesterone biosynthesis (Armstrong and Webb 1997; Monniaux and Pisselet 1992; Spicer *et al.* 1993). Nuclear maturation in the oocytes of cattle (Lorenzo *et al.* 1994) and pigs (Sirotkin *et al.* 2000) has been shown to be enhanced by IGF-I; however, addition of IGF-I was not found to improve nuclear or cytoplasmic maturation of sheep oocytes (Guler *et al.* 2000).

It has been shown that IGFBPs can act to modulate IGF actions in both a positive and negative manner. IGFBPs have higher affinities for IGFs than do the IGF-I and

-II receptors (Jones and Clemmons 1995); therefore, IGF bioavailability can be regulated by the IGFBPs sequestering extracellular IGFs and hence reduce the number of 'free' IGFs available for specific cell surface receptors. This in turn inhibits any mitogenic actions of the IGFs (Jones and Clemmons 1995). Varying binding proteins can regulate IGF actions differently, while the same binding protein can have an IGF-inhibiting or potentiating role under different conditions (Andress and Birnbaum 1992; De Mellow and Baxter 1988; Jones *et al.* 1993). IGFBPs have also been noted to potentiate IGF action. Circulating IGFBPs are believed to prolong the half-life of the attached IGFs by protecting them from degradation, while also acting as a reservoir (Binoux and Hossenlopp 1988; Blum *et al.* 1989) to aid transportation and a controlled delivery of IGFs to a specific cell of tissue (Giudice 1992; Jones and Clemmons 1995). IGFBPs can interact with ECM and somatic cell membranes by attaching themselves to them. This mechanism acts to regulate both IGF and IGFBP action as the maintenance and remodelling of the ECM regulates both binding and release of growth factors from the ECM storage sites. Binding of IGFBPs to the ECM and cell surface molecules depends on specific amino acids motifs within the primary structure of IGFBPs. For example IGFBP-2 contains the integrin recognition sequence RGD (arginine-glycine-aspartic acid), hence it can bind to integrins on the plasma membranes of granulosa and theca cells (Armstrong and Webb 1997). Moreover, it has been suggested that IGFBPs themselves can exert specific cellular effects independently of IGFs and their receptors (Wright *et al.* 2002).

At certain developmental stages of follicle growth, the relative ratio of IGFs to IGFBPs will vary to allow cell-specific growth promoting effects of the IGFs. A mechanism by which facilitation of IGF can be increased is through the activity of specific IGFBP proteases that degrade the IGF/IGFBP complex (Rajaram *et al.* 1997). IGFBP fragments generated by the action of proteases show a marked loss of IGF binding (Collett-Solberg and Cohen 1996). Proteolysis of IGFBPs was first observed in serum from pregnant women, where it was demonstrated that IGFBP-3 was proteolytically cleaved to yield a predominant 29-30kDa form that was still capable of binding IGFs, but with reduced affinity (Giudice *et al.* 1990; Hossenlopp

et al. 1990). Indeed, proteolysis of IGFBPs has been detected in follicular fluid from ewes (Besnard *et al.* 1996b), cattle (Spicer *et al.* 2001), mares (Bridges *et al.* 2002), pigs (Besnard *et al.* 1997) and humans (Mason *et al.* 1996). The degree of proteolytic degradation differs between various follicular compartments and stages of follicle growth.

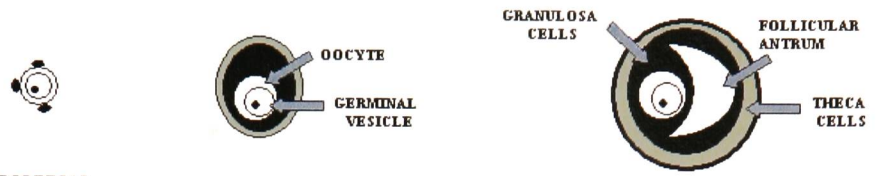
Regulation of IGFs by the IGFBPs appears to be crucial, especially in early follicle growth. This was highlighted when high concentrations of IGF-I were made available to bovine follicles and found to have a detrimental effect on primordial follicle health (Yang and Fortune 2002) and preantral oocyte health *in vitro* (McCaffery *et al.* 2000) and *in vivo* (Armstrong *et al.* 2001). It appears, therefore, that the IGF system plays a vital role in modulating ovarian follicular development and steroidogenesis. During early follicle growth, evidence suggests that IGF-I does not have an important role to play in either granulosa proliferation or as an enhancer of oocyte growth (McCaffery *et al.* 2000; Armstrong *et al.* 2001). Levels of IGFBPs have been found to be high at this stage and therefore limit the access of IGF-I to its specific cell surface receptor. On the other hand, during the later stages of follicle growth levels of IGFBPs fall, thus allowing IGF-I to exert its action (Monget and Bondy 2000). These results imply that regulation of IGF-I is dependent on the developmental stage of the follicle, and that immature follicles have a mechanism whereby high levels of IGFBPs protect them from circulating IGFs.

1.10.3 IGF system in ruminants

The expression of mRNA encoding IGF-II in thecal tissue of bovine ovarian follicles (Figure 1.6) has been detected (Armstrong and Webb 1997) and a similar spatial distribution has been described in sheep (Perks *et al.* 1995). The expression of mRNA encoding IGF-I in ruminants remains controversial. Leeuwenberg *et al.* (1995) detected IGF-I mRNA in ovine granulosal and thecal tissue, and Yuan *et al.* (1998) detected IGF-I mRNA in bovine granulosal cells. By contrast, Perks *et al.* (1995) failed to detect the expression of mRNA encoding IGF-I in ovine follicles. Similarly, Armstrong *et al.* (1998; 2000) failed to detect expression of IGF-I mRNA in bovine follicles (Figure 1.6). In the cow it has been shown that IGF-II is the

principal intrafollicular IGF ligand (Armstrong *et al.* 2000). IGF-II mRNA is expressed in theca cells of bovine follicles at around the time of antrum formation (Armstrong *et al.* 2000), which coincides with the time that theca cells acquire LH receptors. Since IGF-I has been shown to increase LH receptor number in bovine theca cells (Stewart *et al.* 1995), it is hypothesised that *in vivo*, IGF-II takes the place of IGF-I and regulates the timing of onset of LH receptor expression in theca cells via an autocrine mechanism (Armstrong *et al.* 2000).

The two major binding proteins produced by the developing bovine follicle, which regulate the bioavailability of locally produced IGF-II, are IGFBP-2 and IGFBP-4. Expression of mRNAs encoding IGFBP-2 to -5 have been found in bovine follicles (Figure 1.6) and expression of IGFBP-2, -4 and -5 in ovine follicles (Armstrong and Webb 1997; Armstrong *et al.* 1998; 2002; Besnard *et al.* 1996a). IGFBP-2 mRNA has been located in granulosa cells from small and medium-sized healthy antral follicles (1-8mm in diameter). In comparison to these follicles, the proportion of larger healthy (>8mm) antral follicles expressing IGFBP-2 mRNA in their granulosa cells was significantly less. IGFBP-4 mRNA expression has been shown to be restricted to thecal cells of healthy antral follicles, with no difference in expression between small, medium and large follicles (Armstrong *et al.* 1998). In sheep, the expression of mRNA encoding IGFBP-4 and -2 is also restricted to theca and granulosa cells respectively (Besnard *et al.* 1996a).



	PRIMORDIAL FOLLICLE	PREANTRAL	ANTRAL FOLLICLE <1mm-8mm	ANTRAL FOLLICLE >8mm
IGF-I*	-	-	G	CG+G
IGF-II	-	-	T	T
IGFBP-2	-	O+G	O+G	O+CG+G+T
IGFBP-3	-	O	O+G+T	O+G+T
IGFBP-4	-	-	T	CG+T
IGFBP-5	-	-	G	G+T
IGFr	-	O+G+T	O+G+T	O+CG+G+T

Figure 1.6

mRNA expression of the components of the IGF family in developing bovine follicles

Schematic representation of the temporal changes in the expression of mRNA encoding components of the IGF system during bovine follicular development (Armstrong *et al.* 2000; 2002; Nuttinck *et al.* 2004; Roberts and Echternkamp 2003; Webb *et al.* 1999; Yuan *et al.* 1998). IGFr represents type I IGF receptor, O = oocyte, CG = cumulus granulosa cells, G = granulosa cells and T = theca cells. IGF-I* expression remains controversial, with reports both demonstrating its presence (Yuan *et al.* 1998) and absence (Armstrong *et al.* 2000). There is limited data on the expression of type II IGF receptor and insulin receptor in the bovine ovary. Type II IGF receptor mRNA and protein has been localised within granulosa and theca cells of human antral follicles (el Roeiy *et al.* 1993; el Roeiy *et al.* 1994); and ovine granulosa cells of atretic follicles and in theca cells of healthy ovine follicles (Teissier *et al.* 1994). Type II IGF receptors have been found to be present in bovine corpora lutea membrane, however, a fuller investigation into the expression of type II IGF receptors in cattle awaits further study. Insulin receptors are widely distributed throughout ovarian compartments in many animal models and humans (Poretsky *et al.* 1999; Spicer and Echternkamp 1995), and recent preliminary unpublished data has shown the expression of insulin receptor mRNA in the oocyte and granulosa cells of bovine antral follicles (Armstrong D.G. laboratory, Roslin).

The decrease in IGFBP-2 concentration in follicular fluid in cows (Armstrong *et al.* 1998) and sheep (Besnard *et al.* 1996a) during late follicular growth was shown to be due to a loss of expression of mRNA encoding IGFBP-2 in granulosal cells in dominant follicles. Using serum-free bovine granulosa cell cultures, it has been shown that FSH can inhibit the expression of mRNA encoding IGFBP-2 (Armstrong *et al.* 1998). Therefore, it appears that a key step in follicular dominance is the FSH-dependent inhibition of the expression of mRNA encoding IGFBP-2 in granulosa cells. This in turn would increase the available 'free' IGF and hence increase FSH responsiveness of the granulosa cells. In contrast to the expression of mRNA encoding IGFBP-2 in granulosa cells, the expression of mRNA encoding IGFBP-4 in thecal tissue does not change during follicular growth (Armstrong *et al.* 1998; Besnard *et al.* 1996a). In this case, the decrease in IGFBP-4 concentration in follicular fluid during the development of dominance is due to a corresponding increase in the activity of a specific IGFBP-4 protease (Besnard *et al.* 1996a).

The primary regulators of follicular development are the gonadotrophins which act through endocrine mechanisms to control follicle growth. However, to account for the observations that follicles have different fates even when exposed to the same gonadotrophin environment, the concept of intra-ovarian regulation has been developed and is now recognised as a major mechanism regulating follicle development. A limiting factor to realising the full potential of many new reproductive technologies (for example IVF and embryo transfer) is the lack of fertile oocytes. The intraovarian IGF system has been highlighted as a key mechanism involved in regulating oocyte/follicle development. Furthermore, the importance of this system has been highlighted by it's link with the large offspring syndrome which has been observed in bovine offspring following transfer of cloned embryos (Lazzari *et al.* 2002; Niemann and Wrenzycki 2000). IGF-II and IGF type II receptor are imprinted genes that play important roles in preimplantation development and IGF-II expression in bovine cloned embryos has been found to be significantly increased. In nuclear transferred embryos incomplete reprogramming of the donor cell nucleus leads to the abnormal expression of developmentally important genes. However, it is not known whether deviations from the normal

developmental pattern persist throughout fetal development. By investigating IGFs functions, bioavailability and expression patterns throughout follicle growth a more comprehensive description of the components of the IGF system required at various follicle developmental stages will be provided. The role of IGFBP-2 in early stages of follicular development is unknown however it is hypothesised that it could create a barrier for the interaction of IGFs with its receptor on the surface of the oocyte and granulosa cells. IGFs derived from adjacent antral follicles or from the circulation could then bind to IGFBP-2 in the ECM surrounding granulosa cells of immature follicles where it would act as an extracellular store of IGFs. The growth factor could then be accessed by the oocyte and/or its associated granulosa cells when required. The signal to release IGF from the IGF/IGFBP complex would most likely be a specific IGFBP-protease (produced either by the granulosa cells and/or oocyte). This therefore provides a putative mechanism whereby the oocyte can regulate its own exposure, as well as the surrounding granulosa cells, to IGFs and thus control its own development as well as the early stages of granulosa cell proliferation and differentiation. Therefore, the hypothesis behind this work was that the regulation of IGFBP-2 is a major factor controlling early follicle and oocyte development.

1.11 Aims of research

The overall aims of the research detailed in this thesis were:

1. To investigate the effects of IGF-I on early bovine oocyte and somatic cell development and health using a defined serum-free culture system.
2. To analyse temporal and spatial differences in the expression of IGFBPs at different stages of follicle development, as a mechanism to regulating IGF bioavailability.
3. To identify the proteolytic activity of different bovine follicular compartments.

A limiting factor in many reproductive methods is the shortage of fertilisable oocytes. As discussed earlier, superovulation is a process that can increase the number of oocytes, but the response within and between animals is very variable. Methods for *in vitro* maturation (IVM) of oocytes from antral follicles have been developed, but the success rate is low: in a recent study, less than 5% of cow oocytes matured *in vitro*, and developed beyond the 8-cell embryo stage (Chohan and Hunter 2004). This may be because the oocytes are coming from antral follicles that might already be undergoing atresia. It therefore makes sense to grow less developed follicles *in vitro* to a stage where the oocyte can be harvested for subsequent *in vitro* fertilisation (IVF). If we wish reproductive technologies to improve in the near future, it is clearly important to continue research using *in vitro* culture systems as a way of gaining a fuller understanding of the regulatory mechanisms governing follicular development.

The work documented below set out to investigate the effects of insulin-like growth factor-I (IGF-I) on early bovine oocyte and somatic cell development using an *in vitro* serum-free culture system. Chapters 3 and 4 focused on efforts to identify changes in the response and health of a growing follicle at different stages of development in the presence or absence of IGF-I, thereby providing a fuller understanding of the importance of IGF actions throughout early follicular development. The health of follicles was assessed by the measurement of oestradiol

secretion, the degree of pyknosis occurring in the granulosa cells, and analysis of oocyte health.

IGF-I bioavailability, and hence actions, are known to be regulated by their interactions with IGFBPs. Chapter 5 details work aimed at exploring the effects of IGF-I on controlling the expression of IGFBP-2 in maturing follicles. Finally, the proteolytic activity of different components of the bovine follicle were investigated in Chapter 6 in an attempt to elucidate proteases capable of degrading the IGF/IGFBP-2 complex. In Chapter 7 the results of this thesis are summarised and discussed, followed by concluding remarks.

CHAPTER TWO

Materials and Methods

2.1 Bovine ovary collection

Bovine ovaries (Figure 2.2, a) obtained from animals <33 months old, from random stages of the oestrous cycle were obtained from an abattoir and transported to the laboratory. During transit they were maintained at 25-30°C in M199 (HEPES buffered) medium (GIBCO BRL, Life Technologies Ltd., Paisley, UK), supplemented with sodium pyruvate (2mM), glutamine (2mM), Fraction V BSA (cell culture tested) (3mg/ml), penicillin G (75μg/ml) and streptomycin (50μg/ml) (all chemicals from Sigma Chemicals, Poole, UK unless otherwise stated). Upon arrival at the laboratory, the ovaries were placed under a laminar flow hood, rinsed in 70% alcohol and kept warm (37°C) in an incubator.

2.2 Cortical strip and follicle isolation

2.2.1 Dissection medium

Leibovitz's medium (GIBCO BRL, Life Technologies Ltd., Paisley, UK) was used, supplemented with sodium pyruvate (2mM), glutamine (2mM), Fraction V BSA (3mg/ml), penicillin G (75μg/ml) and streptomycin (50μg/ml). Dissection medium was heated to 37°C before use. Leibovitz's medium is buffered by phosphates and free amino acids and maintains a stable balance at room temperature and protects cells from stresses caused by physiological gases present in air.

2.2.2 Isolation of cortical strips from the bovine ovary

Underneath a laminar flow hood, thin transverse slices of ovarian cortex were taken using a scalpel (Figure 2.1 and Figure 2.2, b); slices were 1mm wide and 5mm long. Slices for subsequent culture were placed in dissection medium and maintained at 37°C in an incubator prior to culture. Day 0 sections were fixed overnight in either Bouin's solution (70% v/v picric acid, 25% v/v formaldehyde, 5% v/v glacial acetic acid) or 4% paraformaldehyde (4% v/v paraformaldehyde in dH_2O, 0.1M NaOH, 0.1M Na phosphate pH 7, 4°C) solutions, before being transferred to 70% alcohol for a minimum of 24 hours.

2.2.3 Isolation of early antral follicles

Underneath a laminar flow hood, fine slices (~1mm wide) of ovarian cortex were taken using a scalpel and placed in dissection medium and maintained at 37°C. Early antral follicles were isolated from the cortical slices under a dissecting microscope (Olympus, UK) fitted with a calibrated eyepiece graticule (Graticules Ltd, Kent, UK) using 25 G needles (VWR International Ltd., Leics, UK) attached to syringe barrels. Approximately 50 follicles were isolated on each dissection day (approximately 2 follicle/ovary). Follicles of a normal shape, with an intact basement membrane and no obvious signs of degeneration, were selected for culture (Figure 2.1). The time taken for collection of ovaries from the abattoir to the start of the culture period was a maximum of six hours.

2.3 Cortical strip and follicle culture

2.3.1 Culture medium

The serum-free culture system used was originally developed to induce differentiation of granulosa and theca cells (Campbell *et al.* 1996; Gutierrez *et al.* 1997) and was subsequently adapted for the culture of bovine preantral follicles (Gutierrez *et al.* 2000; McCaffery *et al.* 2000; Thomas *et al.* 2001). McCoy's 5a medium with bicarbonate and HEPES (20mM) was used, supplemented with Fraction V BSA (0.1%), L-glutamine (3mM), penicillin (100IU/ml), streptomycin (0.1mg/ml), transferrin (2.5μg/ml), selenium (4ng/ml), androstenedione (10^{-7} M), insulin (10ng/ml) and L-ascorbic acid sodium salt (50μg/ml), all of which were obtained from Sigma Chemicals, Poole, UK. Both bicarbonate and HEPES buffer systems were used as bicarbonate maintained the most stable balance in the presence of 5% CO_2. However, due to the manipulation of the cultures (changing of culture medium and measurement of follicles) occurring every day or every second day HEPES was present, as it maintains a stable physiological balance at a range of temperatures and protects cells from stresses caused by physiological gases present in air. Bovine Serum Albumin is commonly used in culture media for nutrient transport and also functions to detoxify cell culture by binding to toxic compounds and protects the cells from shear forces in agitated cultures. L-glutamine acting as an

energy source and a precursor for DNA and protein synthesis, penicillin and streptomycin inhibiting bacterial growth, transferrin which is essential for iron transport, selenium which is important in the production of antioxidant enzymes that help prevent cellular damage from free radicals, androstenedione as a substrate for oestradiol production, insulin which is essential for long term cell growth in culture by involvement in stimulation of cell proliferation and cell growth and L-ascorbic acid sodium salt acting as an antioxidant (Thomas *et al.* 2001) were all present in the culture medium.

2.3.2 Cortical strip culture

Cortical strips were individually cultured in 4- or 24-well (flat bottomed) plates (Nalge Nunc International, Hereford, UK, and Corning Inc., NY, respectively) in 500µl of culture medium. Control groups were cultured in culture medium alone, while treatment groups were cultured in culture medium supplemented with the desired treatments (see the materials and methods of individual experiments). Plates were incubated for 3 or 6 days in a sterile humidified air atmosphere with 5% CO_2 at 37°C. Two culture time frames were used as previous work had indicated that the presence of IGF-I may have a detrimental effect on primordial initiation (Yang and Fortune 2002). Cultures were set up to firstly identify if the presence of IGF-I did have a negative effect on primordial activation or early growth and secondly if this were the case could the follicles be rescued after 3 or 6 days of culture by the removal of IGF-I or addition of another factor. Half of the culture medium was replaced, every day, and this conditioned medium was stored at -80°C.

2.3.3 Follicle culture

Follicles were cultured individually in 96-well (V-shaped bottom) plates (Corning Inc., NY) in 250µl of culture medium (Figure 2.1). Control groups were cultured in culture medium alone, while treatment groups were cultured in culture medium supplemented with the desired treatments. Plates were incubated for 2, 4 or 6 days (see the materials and methods of individual experiments) in a sterile humidified air atmosphere with 5% CO_2 at 37°C. Initially, a 4 day culture was set up to investigate if the antral follicles could be maintained with out bursting or undergoing atresia.

Subsequent cultures were set up for 6 days as follicles appeared to be able to be maintained in the culture system successfully for this time frame. In Chapter 5 follicles were cultured for 2, 4 or 6 days to investigate if the expression of IGFBP-2 was effected by day and treatment with IGF-I. Half of the culture medium was replaced every day or every second day (see the materials and methods of individual experiments), and this conditioned medium was stored at -80°C. On the same day that half of the culture media was replaced with fresh culture medium, follicle diameters were measured using a crossed micrometer (basement membrane to basement membrane) under the dissection microscope.

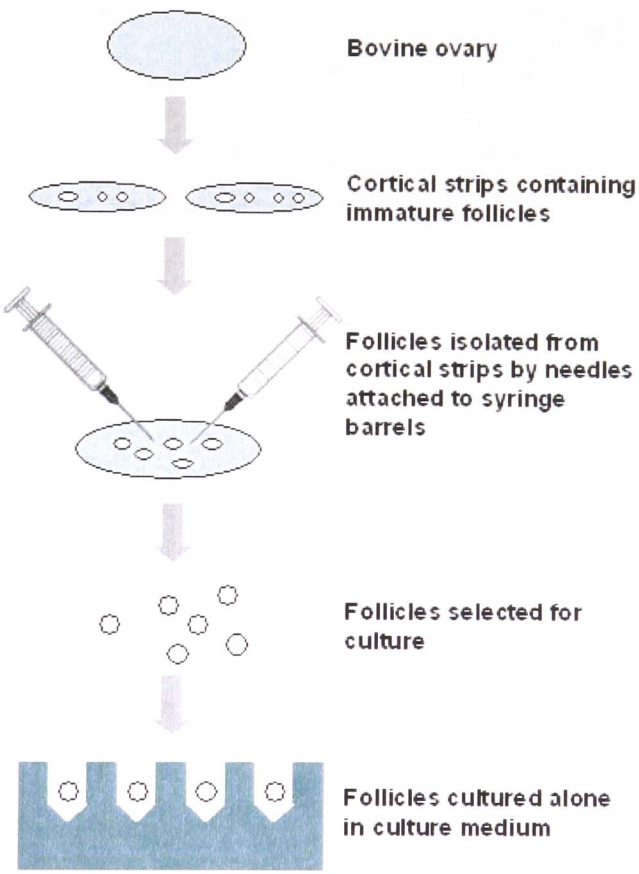


Figure 2.1

Isolation and culture of bovine follicles

2.4 Collection of follicular fluid, mural granulosa cells, oocyte-cumulus complexes and denuded oocytes

Bovine ovaries from random stages of the oestrous cycle were obtained from an abattoir and transported to the laboratory (described in 2.1). Upon arrival at the laboratory, the ovaries were placed under a laminar flow hood, rinsed in 70% alcohol and kept warm (37°C) in an incubator. For each ovary, all visible antral follicles (≥ 3mm in diameter) were aspirated using a syringe connected to a 25 G needle (VWR International Ltd., Leics, UK). Follicular fluid was pooled in 15ml test tubes, left to settle and then the top two-thirds of the follicular fluid was removed and stored at -80°C. The remainder was diluted with dissection medium (see 2.2.1 for method) before being analysed under a microscope. Oocyte-cumulus complexes (OCCs) (Figure 2.2, c) and mural granulosa cells were picked out of the aspirate using a fine glass pipette.

To acquire denuded oocytes (Figure 2.2, d), all oocytes were removed from the aspirate using a fine glass pipette and then exposed to hyaluronidase (300IU/ml) for 10 minutes, before repeated pipetting to ensure the removal of all cumulus cells.

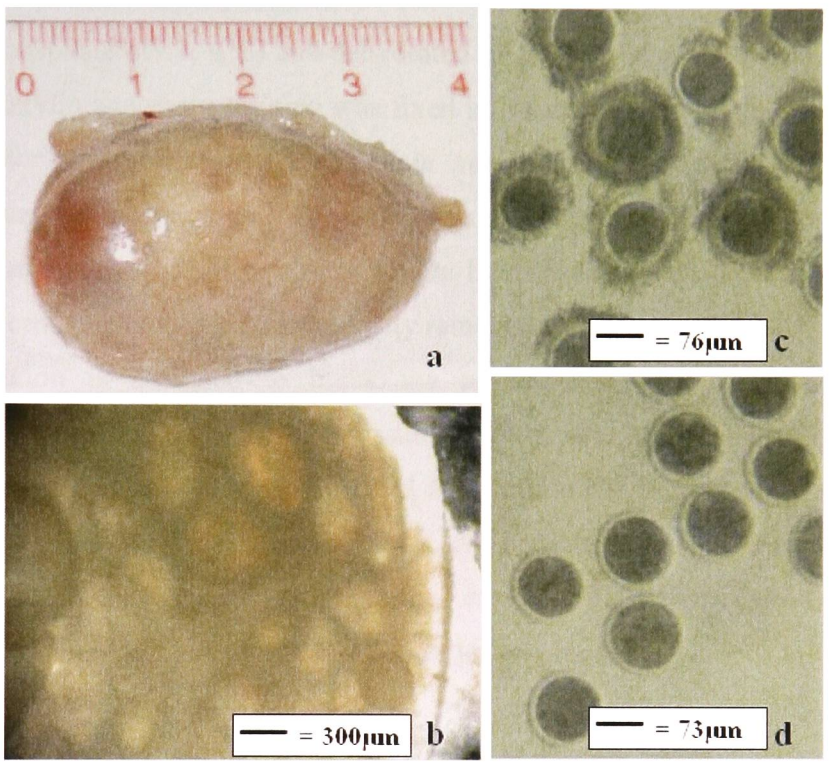


Figure 2.2

Representation of a bovine ovary, follicles and oocytes

Representation of a bovine ovary (**a**), thin bovine ovarian cortical strip containing follicles (**b**), oocyte-cumulus complexes (**c**) and denuded oocytes (**d**).

2.5 Histology

2.5.1 Fixation

At the end of the culture period, cortical strips or follicles were fixed in either Bouin's solution (70% v/v picric acid, 25% v/v formaldehyde, 5% v/v glacial acetic acid) or 4% paraformaldehyde (4% v/v paraformaldehyde in dH_2O, 0.1M NaOH, 0.1M Na phosphate pH 7, 4°C) and dehydrated in ethanol. Material only being used for haematoxylin and eosin staining was fixed in Bouin's solution, whereas material going on for immunocytochemistry or *in situ* hybridisation was fixed in 4% paraformaldehyde solution. Paraformaldehyde was used as a fixative for material going on for immunocytochemistry or *in situ* hybridisation because unlike Bouins, the protein cross-links formed can be easily removed by citrate buffer, as this sodium citrate solution is designed to break the protein cross-links, therefore easily unmasking the antigens within the tissue sections, and this enhancing staining intensity of antibodies. Material was fixed for a minimum of 5 hours before being placed in 70% ethanol for at least 24 hours. All concentrations of ethanol used for processing of follicles had a small amount of eosin (VWR International Ltd, Leics, UK) added to aid visualisation of the follicles during processing.

2.5.2 Processing of tissue for microscopic examination

2.5.2.1 Processing of samples

Material for histological assessment was dehydrated by a series of changes achieved through increasing ethanol concentrations. Absolute alcohol ethanol was replaced with cedar wood oil (VWR International Ltd., Leics, UK) for a minimum of 24 hours. The oil was then cleared from the cortical strips or follicles with toluene (in a fume hood) for 30 minutes. Cortical strips or follicles were embedded in paraffin wax (VWR International Ltd., Leics, UK) at 60°C, with changes every hour for 4 hours to remove all traces of toluene.

2.5.2.2 Sectioning and mounting

The samples were sectioned at 6μm with a microtome (Leica Microsystems, Milton Keynes, UK). The sections were then floated out in a water bath at 42°C and mounted on Superfrost plus microscope slides (VWR International Ltd., Leics, UK). Slides were left to dry overnight at 37°C, before either staining with haematoxylin and eosin or being used for immunocytochemistry or *in situ* hybridisation.

2.5.2.3 Haematoxylin and eosin staining

Haematoxylin stains the nucleus purple and eosin stains the cytoplasm pink. Firstly, sections are dewaxed in xylene for 15 minutes and taken down through a series of ethanol concentrations (absolute to 70% v/v). The staining procedure was as follows (for reagent preparations see Dury and Wallington 1976). Bouin's fixative was removed from sections by dipping slides in 70% ethanol with lithium carbonate. Slides were rinsed in tap water and placed in haematoxylin (5 minutes), rinsed in water again and placed in Scott's tap water substitute. Sections were rinsed in tap water and placed in Eosin (2minutes, 1:1 solution), dipped in tap water and placed in potassium alum (VWR International Ltd., Leics, UK) (3 minutes), and rinsed again in tap water. The sections were dehydrated through a series of ethanol concentrations and placed in xylene. The sections were mounted using DPX mounting medium (VWR International Ltd., Leics, UK) and a glass cover slip.

2.5.2.4 Histological assessment

Histological measurements and observations were made under the light microscope with a crossed micrometer (Graticules Ltd., Kent, UK). The section containing the oocyte nucleolus – or if this was absent, the largest cross-section of the oocyte – was used for observations and measurements. Oocyte health was analysed according to the following criteria: '0' indicating an absent or severely misshapen oocyte with no germinal vesicle and obviously degenerate; '1' indicating a misshapen oocyte; and '2' indicating a morphologically normal oocyte with an intact germinal vesicle (McCaffery *et al.* 2000) (Figure 2.3, b and d). Follicles with oocytes graded as 0 or 1 were grouped together and termed degenerate oocytes. The number of degenerate oocytes was then expressed as a percentage of the total oocytes. Oocyte and follicle

diameters were measured and the oocyte:follicle ratios calculated. Counting the number of pyknotic and non-pyknotic granulosa cells present, and then expressing them as a mean percentage of pyknotic granulosa cells for each treatment group, measured granulosa cell death (Figure 2.3, a and c).

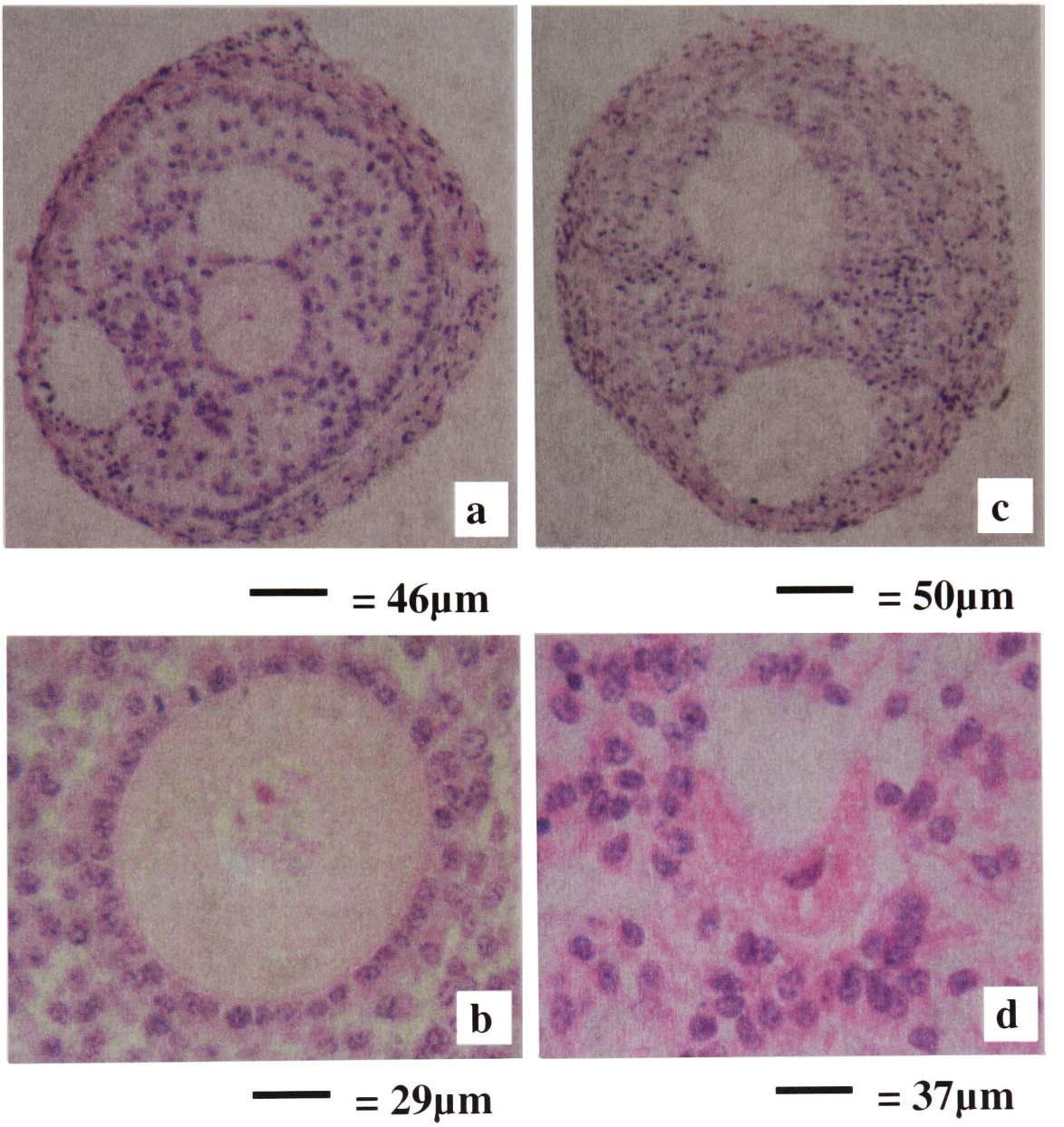


Figure 2.3

Histological sections representing healthy and atretic follicles

a = a follicle with a healthy oocyte and granulosa cells, as well as an antrum; **b** = a healthy oocyte; c = a follicle with an unhealthy oocyte and pyknotic granulosa cells; and **d** = an unhealthy oocyte.

2.6 Immunocytochemistry

2.6.1 Detection of IGFBP-2 immunoreactivity in bovine follicles

See materials and methods for Chapter 5.

2.6.2 Detection of androgen receptor immunoreactivity in bovine follicles

See materials and methods for Chapter 3.

2.7 Detection of IGFBP-2 by *in situ* hybridisation

See materials and methods for Chapter 5.

2.8 Measurement of oestradiol in culture medium using a delayed enhancement lanthanide fluorometric immunoassay (DELFIA)

At the end of the culture period, all the conditioned culture medium which had been collected every day or every second day (see the material and methods of individual experiments) was taken to Roslin Institute were detection of oestradiol by the DELFIA procedure as previously described by Thomas *et al.* (2003a) was performed by Charis Hogg, Karen Troup and Elaine McCullough. Medium from the three treatment groups in all size ranges of follicles was analysed for oestradiol content. Oestradiol was biotinylated by standard procedures using 17β-oestradiol-3-(O-carboxy) methylether and EZ-Link biotin hydrazise (Pierce Warriner UK Ltd). Nunc-Immuno Maxisorp 96-well plates were coated with donkey anti-sheep serum by incubating overnight at 4°C in the presence of donkey anti-sheep serum (250μg/ml) made up in bicarbonate buffer pH 9.6 (100μl/well). The primary antibody was raised in sheep against 17β-oestradiol 6-(o-carboxymethy)-oxime: BSA (Webb *et al.* 1985). Biotinylated oestradiol, follicle conditioned medium, oestradiol standards and a 1:50,000 dilution of primary antibody, made up in 200μl of assay buffer, were added to pre-coated wells of the microtitre plate. The assay buffer consisted of Tris buffer (50mmol/l; pH 7.8) supplemented with sodium chloride (150mmol/l), bovine gamma globulin (1%, w/v), Tween-20 (0.01%; v/v), thimerosal (0.0008%; w/v) and diethylenetriamine-penta-acetic acid (0.1moles/l). After incubating the plates overnight at 4 °C, they were washed (4x) in a wash buffer consisting of Tris buffer

(50mmol/l; pH 7.8) supplemented with sodium chloride (150mmol/l), Tween-20 (0.01%; v/v) and thimerosal (0.0008%; w/v), before adding 100µl of assay buffer containing 100ng/ml europium labelled streptavidin (Perkin-Elmer Life Sciences, UK), followed by incubation at room temperature for 1 hour with shaking. The plates were washed (4x) in wash buffer before addition of 200µl of DELFIA enhancement solution (Perkin-Elmer Life Sciences) to each well of the microtitre plate, and incubated for a further 5 minutes with shaking at room temperature. The plates were analysed on a Victor 2 Multilabel Counter (Perkin-Elmer Life Sciences) by time resolved fluorimetry. The emission and excitation wavelengths were 615nm and 340nm respectively, with a time delay of 400µs. The inter- and intra-assay coefficients of variation were 13.2 and 9.6% respectively. The minimum detectable level was 8.5pg oestradiol per 250µl.

2.9 Analysis of IGFBP-2 proteolytic degradation in conditioned media

Immunoblotting and western ligand blotting protocols were adapted from the procedures previously described by Monget *et al.* (2003). To optimise conditions a series of electrophoresis of proteins and blotting procedures were carried out using the NuPAGE Novex Pre-Cast Gel System (Invitrogen Ltd., Paisley, UK). Initially reducing conditions were used which meant that the disulphide bonds within IGFBP-2 would be broken down into subunits. However, problems occurred with detection of the protein so non-reducing conditions were used as only nonconvalent interactions are destroyed using this technique, thus the protein is linearises but the basic structure is maintained. Samples were run on gels with various gradients to optimise separation of proteins and resolution of the protein bands. Problems with excessive background, hence low visualisation of protein bands, were alleviated by the use of a range of blocking agents.

2.9.1 Immunoblotting

Recombinant bovine IGFBP-2 (31 kDa) (1ng/ml) (GroPep Limited, Adelaide, Australia) and oocyte-cumulus complexes (2.5 oocytes/µl), denuded oocytes (2.5 oocytes/µl), granulosa cells, bovine follicular fluid (positive control), intact IGFBP-2 alone (negative control) or concentrated conditioned media (see section 6.2.4) (final

volume 10µl) were incubated for 20 hours at 37°C, in a sterile humidified air atmosphere with 5% CO_2. 2.5 oocytes/µl were used as initial studies using 1 oocyte/µl fail to cleavage IGFBP-2 hence to account for the possibility of a dilution effect occurring the concentration of oocytes/µl was increased. The final volume used was 10µl, so 25 oocytes were added. SeeBlue Plus2 Pre-stained Standard marker was run to allow easy visualisation of protein molecular weight ranges during electrophoresis and evaluation of western transfer efficiency (Invitrogen Ltd., Paisley, UK).

Samples were submitted to electrophoresis on a 12% NuPAGE Bis-Trs SDS-polyacrylamide gel with NuPAGE MOPS running buffer, under nonreducing conditions at 200V for 50 minutes. The gel was then washed in NuPAGE transfer buffer for 30 minutes (all NuPAGE items obtained from Invitrogen Ltd., Paisley, UK). The proteins were then electrotransferred onto nitrocellulose membranes (Hybond ECL, 0.2µm pore size) (Amersham Biosciences UK Ltd, Bucks, UK) using 30V for 1 hour. Nitrocellulose membranes were treated for 2 hours at room temperature with TBS (20mM Tris and 137mM NaCl, pH 7.6) containing 10% non-fat dry milk (NFDM) and 0.2% Tween-20 to saturate non-specific sites. Thereafter, membranes were incubated for 1 hour at 37°C in TBS containing 5% (NFDM), 0.05% Tween-20, and antibodies against IGFBP-2 (rabbit anti-bovine IGFBP-2 antiserum, Upstate Biotechnology Incorporated, Lake Placid, NY) (dilution 1/2000) (Sigma Chemicals, Poole, UK). Once washed in TBS containing 1% (NFDM) and 0.2% Tween-20, nitrocellulose filters were incubated for 1 hour at 37°C with a goat anti-rabbit IgG antibody coupled with horseradish peroxidase (dilution 1/5000) (Sigma Chemicals, Poole, UK). After washing, the signal was revealed by chemiluminescence detection (Figure 2.4).

2.9.2 Western ligand blotting

After electrophoresis and electrotransfer of proteins as described in 2.9.1, nitrocellulose filters were washed for 2 hours with PBS (1.3M NaCl, 0.07M Na_2HPO_4, 0.03M NaH_2PO_4 10x solution diluted to1x before use, pH 7.4) containing 10% (w/v) BSA and 0.2% (v/v) Tween-20 to saturate non-specific sites. Thereafter,

membranes were incubated overnight at 4°C in PBS containing 5% (w/v) BSA, 0.05% (v/v) Tween-20, and biotinylated IGF-II (dilution 1/1250) (IGF-II, Sigma Chemicals, Poole, UK and Biotin Protein Labelling Kit, Roche Diagnostics Ltd., Lewes, UK). Afterwards, membranes were washed with PBS containing 1% (w/v) BSA and 0.2% (v/v) Tween-20 and incubated for 1 hour with extravidin peroxidase (Sigma Chemicals, Poole, UK) in PBS containing 0.2% (v/v) Tween-20 (dilution 1/3000). After washing, the signal was revealed by chemiluminescence detection (Figure 2.4).

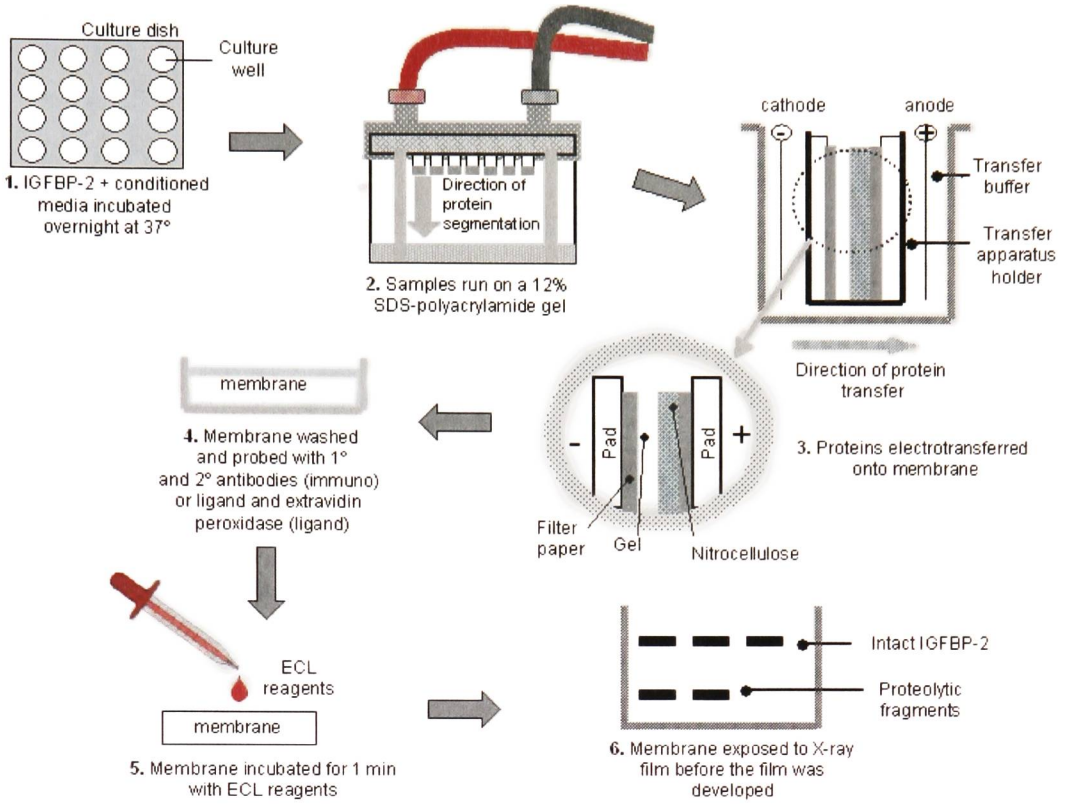


Figure 2.4

Detection of proteolysis by electrophoresis and immuno and ligand blotting

CHAPTER THREE

The Effect of IGF-I Either Alone or Interacting With Androstenedione on Primordial Follicle Initiation and Early Follicle Growth

3.1 Dosage effect of IGF-I on primordial follicle activation and the early development of bovine follicles

3.1.1 Introduction

Despite advances in the understanding of the mechanisms involved in different stages of follicle development, the factors regulating initiation and early follicle growth remain only partially elucidated. It is generally accepted that primordial follicles do not proliferate after birth, so when the follicle pool is exhausted the female is considered to be infertile. Therefore, the correct order and completion of events that occur during the transition from primordial to primary follicle stages is crucial to maintaining female fertility. The primary regulators of follicle growth and development have been shown to be the gonadotrophins, FSH and LH; however, the early stages of development are gonadotrophin-independent (Awotwi *et al.* 1984; Gong *et al.* 1996). Therefore, other factors must be responsible for controlling follicle growth initiation.

A great deal of discussion has centred on whether initiation is regulated by a release from the effects of inhibitory factors or by a stimulatory signal. A variety of factors have been implicated in the activation of primordial follicles such as kit ligand (KL) (Parrott and Skinner 1999), basic fibroblast growth factor (bFGF) (Nilsson *et al.* 2001), bone morphogenetic protein-4 (BMP-4) (Nilsson and Skinner 2003), leukemia inhibitory factor (LIF) (Nilsson *et al.* 2002), insulin (Kezele *et al.* 2002b) and nerve growth factor (NGF) (Dissen *et al.* 2001). The tyrosine kinase receptor c-kit and its ligand, kit ligand, have been localised to oocytes and granulosa cells respectively (Motro and Bernstein 1993). KL has been shown to promote initiation and progression of follicle development in the rat ovary, as inhibition of KL actions by a c-kit antibody (ACK-2) was shown to completely block primordial follicle development (Parrott and Skinner 1999). In a similar manner bFGF, BMP-4 and LIF were also demonstrated to induce primordial follicle development, while neutralising antibodies reduced the proportion of spontaneous developing primordial follicles. Furthermore, it was shown that LIF may also interact with KL to increase the primordial to primary follicle transition (Nilsson *et al.* 2001; 2002; Nilsson and

Skinner 2003). $NGF^{-/-}$ mice have a reduction in the number of primary follicles present in their ovaries compared to wild-type ovaries (Dissen *et al.* 2001). It has been suggested that NGF may be important for the initial differentiation of the flattened pre-granulosa cells of primordial follicles into the enlarged, cuboidal shape that characterises these cells in primary follicles. If this is the case, then NGF is crucial during the time period immediately preceding the initiation of follicular growth (Dissen *et al.* 2001). Vascular endothelial growth factor (VEGF), which is a key regulator in angiogenesis in the ovary and known to be produced by theca and/or granulosa cells, has been shown to significantly stimulate the number of primary and secondary follicles in a dose-dependent manner, suggesting an increase in the number of primordial follicles being recruited from the primordial pool into the growing phase (Danforth *et al.* 2003). Furthermore, growth differentiating factor-9 (GDF-9), which is a member of the TGF-β superfamily, has been shown in humans (Hreinsson *et al.* 2002), but not other species (Nilsson and Skinner 2002), to significantly increase the proportion of primordial follicles initiating growth in culture (Hreinsson *et al.* 2002). Conversely, anti-Müllerian hormone (AMH) [also known as müllerian inhibitory substance (MIS)] has been shown to have the opposite role, functioning as an inhibitory growth factor in the ovary during early stages of follicle growth. Examination of AMH-null mice revealed lower numbers of primordial follicles and more growing follicles compared with wild-type litter mates (Durlinger *et al.* 1999). In addition, AMH has been shown to inhibit primordial follicle initiation, as mouse ovaries cultured in the presence of AMH were found to contain 40% fewer growing follicles when compared with control ovaries (Durlinger *et al.* 2002).

Once follicles have been initiated to grow they can respond to varying levels of endocrine, paracrine and autocrine factors. It is well established that gonadotrophins (FSH and LH) are not required for the development of preantral follicles but are essential for the progression to the antral stages (Awotwi *et al.* 1984; Gong *et al.* 1996). Nevertheless, work has provided evidence that gonadotrophins can affect the rate of development of preantral follicles in mice (Mizunuma *et al.* 1999), rats (McGee *et al.* 1997), humans (Wright *et al.* 1999) and sheep (Campbell *et al.* 2004).

Therefore, although FSH is not essential, under optimum conditions for follicle growth it may be beneficial. As early follicular development can be induced in the absence of FSH, it is now widely accepted that intraovarian factors are the main regulators during these early stages. Activin, an intraovarian factor produced from secondary follicles, has been shown to inhibit early follicle growth by causing small preantral follicles to remain in a dormant state until a time when activin levels decline (Mizunuma *et al.* 1999). Interestingly, the effect of activin A has been found to be developmentally dependent with it having a stimulatory effect on preantral follicles from immature mice, yet no effect on follicle growth in preantral follicles from adult mice (Yokota *et al.* 1997). On the other hand, there are many factors that have been confirmed as having a stimulatory effect on early and preantral follicle development. GDF-9 is an oocyte-derived factor that is important for further follicle development and in rat ovaries the progression of early primary (stage 1) follicles to stage 2 primary follicles (Nilsson and Skinner 2002). Furthermore, GDF-9 has been shown to stimulate preantral follicle growth in mice (Hayashi *et al.* 1999), and improve human follicle viability and promote progression of early follicles to the secondary stage (Hreinsson *et al.* 2002). Recently, VEGF has been found to stimulate preantral follicle growth in a time- and dose-dependent manner (Danforth *et al.* 2003). Keratinocyte growth factor (KGF), also known as fibroblast growth factor-7, is yet another factor that has been discovered to play a role in early stages of follicle development. Treatment of rat preantral follicles with KGF promoted their survival, growth and differentiation (McGee *et al.* 1999). MIS (also known as AMH) has been shown to inhibit mouse primordial follicle initiation (Durlinger *et al.* 2002). However, when rat preantral follicles were cultured in the presence of MIS, it was found to enhance the effects of FSH both on follicle diameter and cell number (McGee *et al.* 2001). Interestingly, MIS was not found to have an effect on preantral follicle cell differentiation (McGee *et al.* 2001).

The extracellular matrix (ECM) exerts stringent control over cell differentiation, either by inducing specific signals via integrins or by modifying cell responsiveness to growth factors and hormones (Giancotti 1997). Hence, growth factors that are ligands that bind to the ECM must interact with components of the ECM; this

interaction will in turn have an effect on cell growth, differentiation and survival. The transition of a mouse follicle from a primary to a multi-layer stage has been shown to be enhanced by the interaction of activin A with the ECM component laminin, yet suppressed by the interaction of activin A with another ECM component, collagen (Oktay *et al.* 2000).

Insulin-like growth factor-I (IGF-I) knock-out mice exhibit a block in follicle development at the early antral stage (Baker *et al.* 1996) suggesting that IGF-I is expendable for primordial initiation and preantral growth. *In vitro* culture of rat ovaries (Kezele *et al.* 2002b) and bovine cortical strips (Derrar *et al.* 2000; Yang and Fortune 2002) showed that IGF-I did not promote the primordial to primary follicle transition, further supporting the view that IGF does not play a stimulatory role in the early stages of follicle development. In addition, in the culture system used by Yang and Fortune (2002) IGF-I was found to have a dose-dependent negative effect on bovine primordial follicle activation and health. However, studies have detected IGF-I binding in bovine preantral follicles (Wandji *et al.* 1992), and identified type I IGF receptor in oocytes, granulosa and theca cells as being present from the preantral stage in bovine follicles (Armstrong *et al.* 2002). IGF receptors are also known to be present in the oocytes and granulosa cells from the primordial stage, as well as in theca cells from the preantral stage in human follicles (Qu *et al.* 2000). The percentage of follicles in the primary stage of development was shown to increase when human ovarian cortex was cultured for two weeks in the presence of IGF-I and IGF-II (Louhio *et al.* 2000). Additionally, IGF-I has been found to have a beneficial effect on preantral follicle development in rats (Zhao *et al.* 2001) and cattle (Gutierrez *et al.* 2000; Itoh *et al.* 2002). Thus, these findings indicate that the IGF system is still a potential candidate for regulating follicle initiation and/or early follicle growth.

The aim of the following experiment was to investigate the importance of IGF-I in regulating follicle initiation and/or early follicle growth in the bovine ovary, by culturing bovine ovarian cortical strips in the presence of IGF-I. In addition to just investigating the effects of IGF-I during early follicle development, thorough

analysis was carried out to account for discrepancies often observed when comparing different studies on primordial follicle initiation. A problem associated with studying follicle activation is that the criteria used to classify follicles to a particular developmental stage often vary between studies or are unclear. The values obtained can then be analysed by looking at percentage or total numbers, which further add to the confusion when comparing between studies. Therefore, in this current study a clear method for classification has been set out and the data has been analysed by comparing both percentage and total numbers of follicles.

3.1.2 Materials and methods

3.1.2.1 Cortical strip isolation and culture

Cortical strips were isolated from bovine ovaries and cultured individually for 3 or 6 days, as described in Chapter 2.

3.1.2.2 Treatments

Culture medium (control) was prepared as described in 2.3.1, but no androstenedione was added to the McCoy's culture medium. Androstenedione was removed from the culture medium as studies carried out on ovine preantral follicles had indicated that although androstenedione was found to increase follicular differentiation it decreases oocyte survival. It was hypothesised that androstenedione present in the culture medium may have been over-stimulating the immature follicles and thus causing deleterious effects on oocyte development (Thomas *et al.* 2002). Although IGF-II is the principal intrafollicular IGF ligand in cattle (Armstrong *et al.* 2000), IGF-I was used to allow comparison with previous studies and because it has been hypothesis that *in vivo* IGF-II will have similar effects as IGF-I *in vitro* (Armstrong *et al.* 2000). Human recombinant IGF-I (HR IGF-I), which does bind to IGFBPs, was purchased from Sigma Chemicals, Poole, UK. Two doses of HR IGF-I were used, a low dose of 10ng/ml and a high dose of 100ng/ml. The low dose of 10ng/ml represented a physiological dose that would be able to bind to the IGFBPs and would therefore be regulated by the level of IGFBPs present. The high dose of 100ng/ml, on the other hand, would swamp the IGFBPs present, allowing IGF-I to bypass any regulatory mechanism of the IGFBPs and hence bind freely to IGF receptors. The analogue Long R^3 IGF-I (LR3 IGF-I), which does not bind to binding proteins, was purchased from Sigma Chemicals, Poole, UK. LR3 IGF-I will, therefore, bypass any IGFBP regulatory mechanism and go straight to the IGF receptors to have a direct effect on the target cell.

Treatment groups and 'n' numbers were as follows ('n' number values represent the number of bovine cortical strips cultured for each treatment group for 3 and 6 day cultures respectively):

Treatment group	'n' numbers for 3 day culture	'n' numbers for 6 day culture
A Day 0	12	12
B Control	6	6
C 5ng/ml LR3 IGF-I	6	6
D 10ng/ml HR IGF-I	6	6
E 100ng/ml HR IGF-I	6	6

Table 3.1.1

Treatment groups and number of replicates

3.1.3 Follicle classification

Follicles were measured and characterized with reference and modifications to the Braw-Tal and Yossefi (1997) and McNatty et al. (1999) classification of small bovine and ovine follicles respectively.

Follicle (type)	Layers of granulosa cells	Total no. of granulosa cells	Oocyte diameter (μm)	Follicle diameter (μm)	Presence of theca interna (%)
Primordial (Type 1)	1 (all flattened)	<10	<30	<40	0
Transitory (Type 1a*)	1 (one cuboidal)	<10	<30	<40	0
Transitory (Type 1a)	1 (mixture of flattened and cuboidal)	10-40	<30	<40	0
Primary (Type 2)	1-2 (all cuboidal)	10-40	<50	40-80	35
Small preantral (Type 3)	3	41-100	<70	81-130	100
Large preantral (Type 4)	4-6	101-250	<90	131-250	100
Small antral (Type 5)	>6	>250	<90	250-500	100

Table 3.1.2

Classification and characterisation of small bovine follicles

In this classification system for small bovine follicles (Table 3.1.2), the follicles are classified as types 1-5. Type 1 refers to primordial follicles (oocyte surrounded by flattened granulosa cells); type 1a* refers to transitory follicles (oocyte surrounded by flattened granulosa cells and one cuboidal granulosa cell); type 1a refers to transitory follicles at a later stage of growth than 1a* follicles (oocyte surrounded by a mixture of flattened and cuboidal granulosa cells); type 2 are primary follicles (oocyte surrounded by one or two layers of cuboidal granulosa cells); type 3 are known as small preantral follicles (three layers of granulosa cells); type 4 are large preantral follicles (four or more layers of granulosa cells); and type 5 are small antral follicles (more than five layers of granulosa cells and early antrum formation).

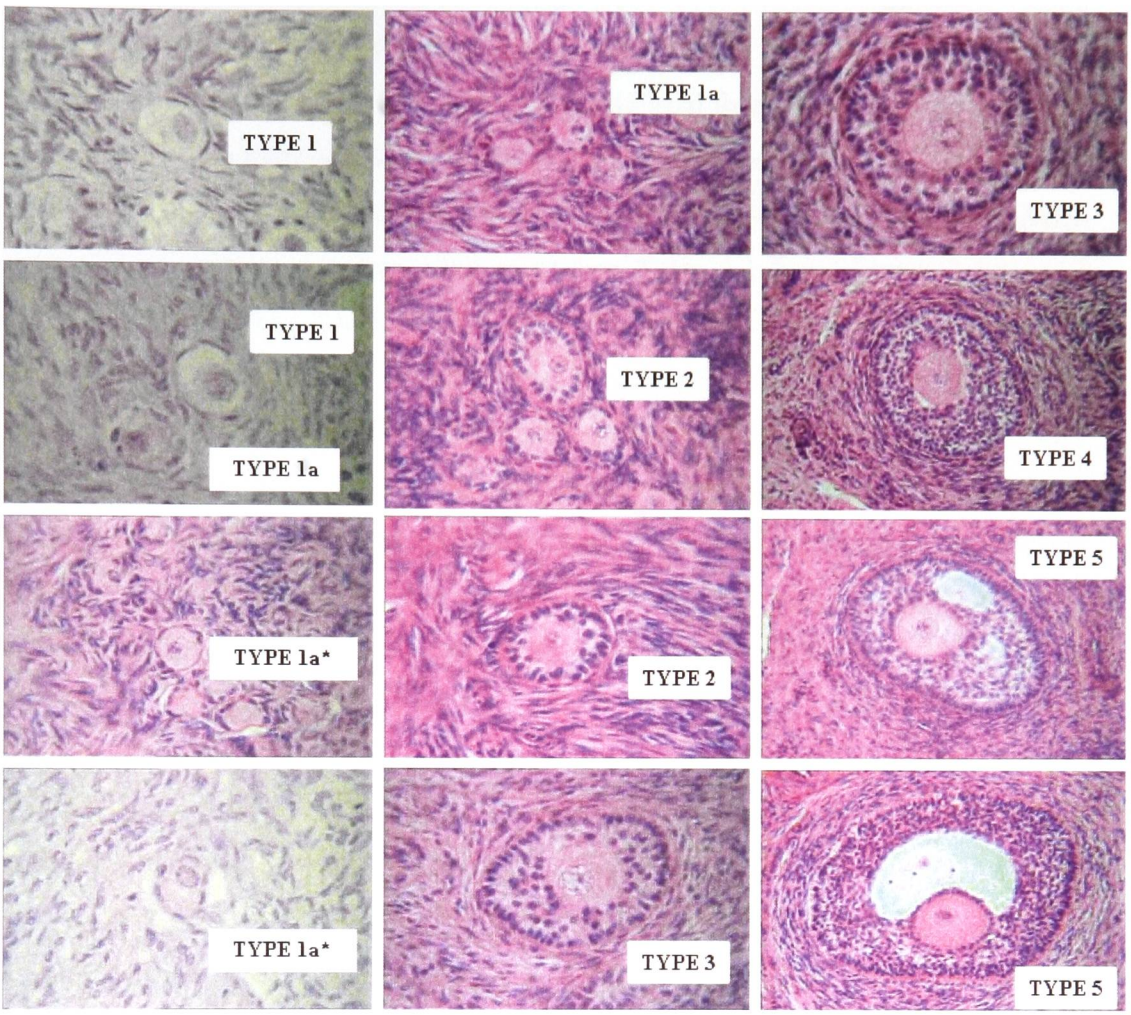


Figure 3.1.1

Histological representation of bovine follicles at different stages of development

In this classification system for small bovine follicles, the follicles are classified as types 1-5. Type 1 = primordial follicle, type 1a* = transitory follicle, type 1a = transitory follicle at a later stage of growth than 1a* follicle, type 2 = primary follicle, type 3 = small preantral follicle, type 4 = large preantral follicle and type 5 = small antral follicle.

3.1.4 Histological assessment

Cortical strips were fixed in paraformaldehyde, processed for histology and mounted on slides at the end of the culture period, as described in Chapter 2. Samples were sectioned at 6μm, and every fifth section was analysed throughout the whole of each cortical strip. The total number of follicles present was counted on each section. To avoid double counting, only follicles with a visible nucleolus were counted. Follicles were classed by their developmental stage (see Table 3.1.2 and Figure 3.1.1), and oocyte health [growing (type 1a* onwards) follicles only] was analysed by grading the oocyte of each follicle as unhealthy (a degenerate germinal vesicle and/or misshapen oocyte) or healthy (a morphologically normal oocyte with an intact germinal vesicle). Primordial oocyte health was not analysed in this current study as the culture system caused wholesale activation. Therefore, after culture most strips had very few or no primordial follicles left, which meant a valid investigation on the effects of IGF-I on primordial oocyte health could not be carried out.

3.1.5 Statistical analysis

The mean percentage of follicles at each stage of development per cortical strip and the mean number of follicles per cortical strip for each treatment group were calculated for the following developmental stage categories: non-growing (type 1), growing (type 1a* and 1a) and advanced (type 2 onwards). The data set was normally distributed in accordance with the Anderson-Darling test, variances were homogeneous and treatment groups were randomly assigned, therefore, a powerful parametric test could be used to analysis samples. ANOVA and t-tests were used as they are both very robust statistical tests. Mean percentages of follicles per cortical strip and the mean number of follicles per cortical strip were compared between experimental groups using one-way ANOVA, with subsequent two-sample t-tests to allow for individual comparisons between groups.

The difference between treatment groups in the mean percentages of degenerate oocytes per cortical strip, within the growing follicles, was compared using one-way ANOVA, with subsequent two-sample t-tests to allow for individual comparisons between groups.

3.1.6 Results

3.1.6.1 Follicle development

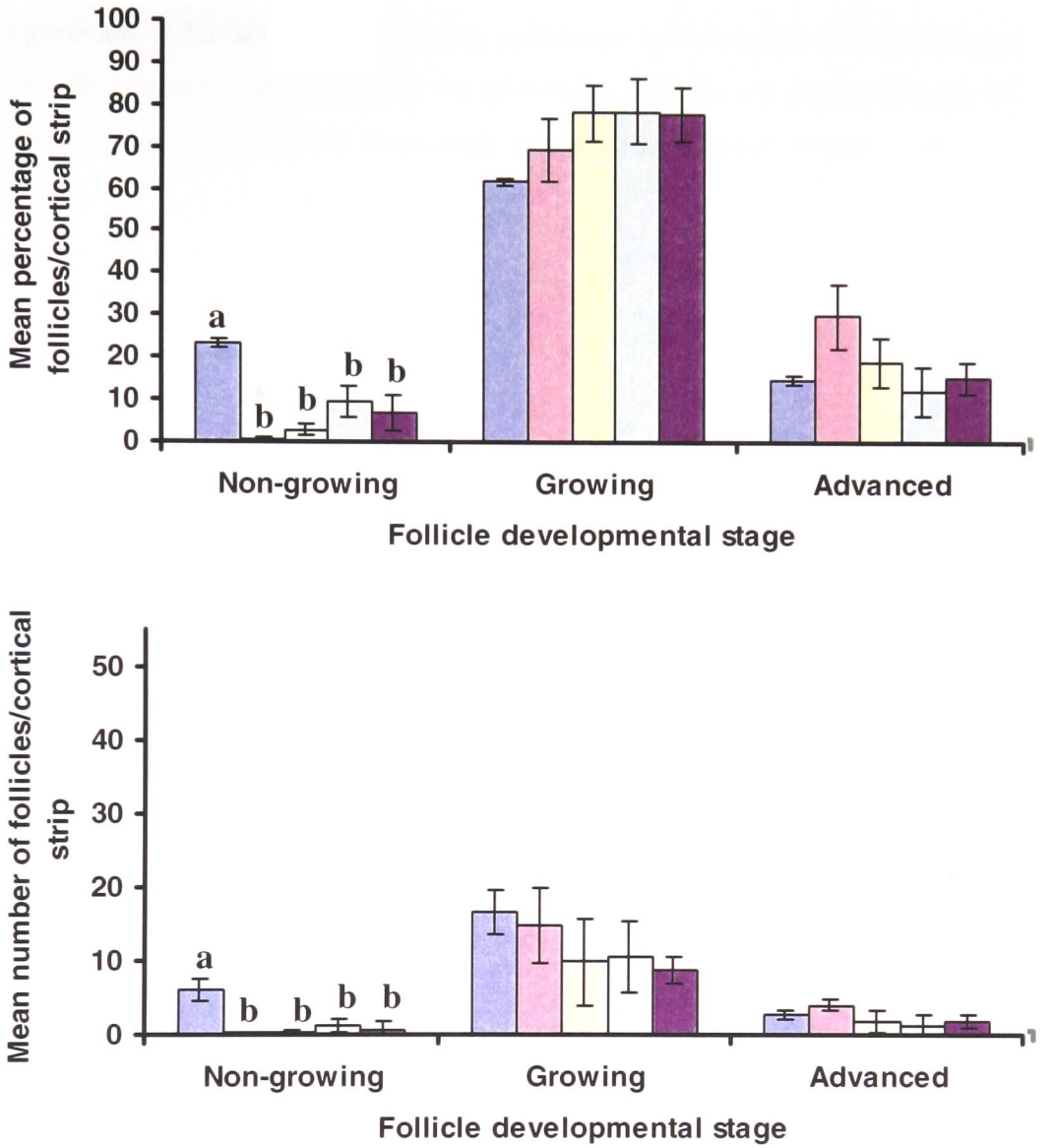


Figure 3.1.2

Effect of IGF-I on primordial follicle initiation and early follicle development after 33 days of culture

Distribution of follicles in pieces of bovine ovarian cortex after 3 days of culture.

Values are the mean percentage or number of follicles per cortical strip per treatment group ± SEM for 4 cultures.

Day 0 = (n=12), Control = (n= 6), 5ng/ml LR3 IGF-I = (n=6), 10ng/ml HR IGF-I = (n=6) and 100ng/ml HR IGF-I = (n=6).

In all treatment groups there was a significant decrease in the mean percentage and mean number of follicles per cortical strip compared to Day 0, within the non-growing developmental stage category ($p<0.05$) (Figure 3.1.2). However, there was no significant difference in the growing population between any of the treatment groups. This therefore indicates that the primordial follicles are not growing to the later stages of development but are perhaps being lost to follicular atresia.

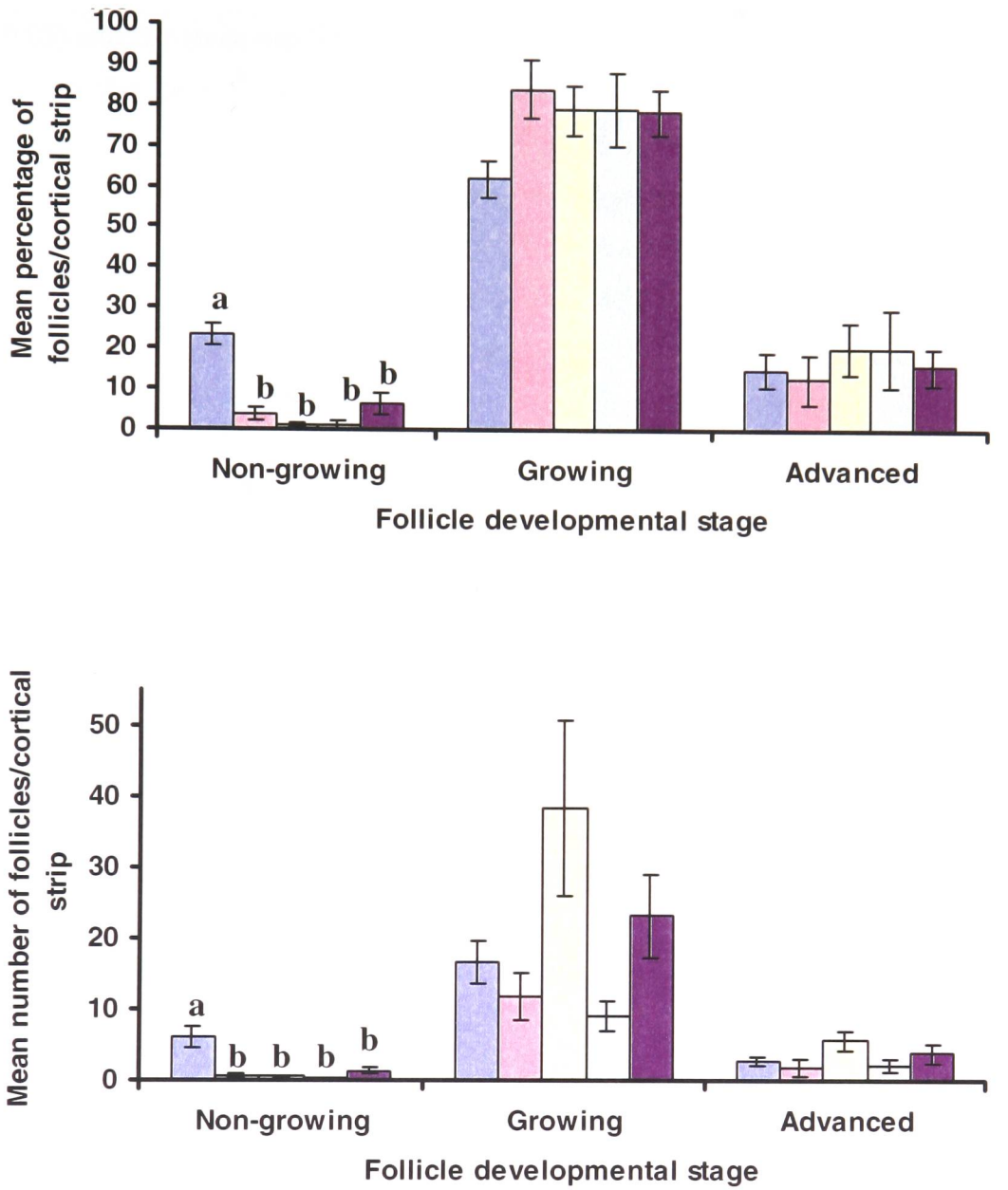


Figure 3.1.3

Effect of IGF-I on primordial follicle initiation and early follicle development after 6 days of culture

Distribution of follicles in pieces of bovine ovarian cortex after 6 days of culture.

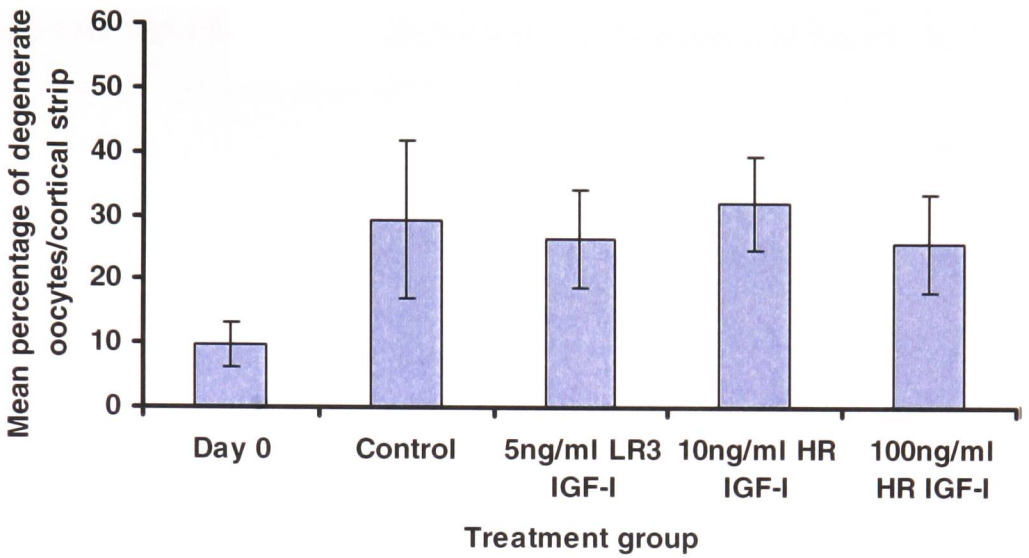
Values are the mean percentage or number of follicles per cortical strip per treatment group ± SEM for 6 cultures.

Day 0 = (n=12), Control = (n= 6), 5ng/ml LR3 IGF-I = (n=6), 10ng/ml HR IGF-I = (n=6) and 100ng/ml HR IGF-I = (n=6).

In all treatment groups there was a significant decrease in the mean percentage ($p<0.05$) and the mean number ($p<0.01$) of follicles per cortical strip compared to Day 0, within the non-growing developmental stage category (Figure 3.1.3). A significant difference in the mean number of follicles per cortical strip was found between treatment groups in the growing developmental stage category when compared using ANOVA ($p<0.05$) (Figure 3.1.3). However, when subsequent two-sample t-tests were carried out (which is a more stringent test than ANOVA) to identify individual comparisons between groups, no two groups were significantly different with a p value of ≤ 0.05. However, comparisons between Day 0 and LR3 IGF-I and control and LR3 IGF-I both revealed p values of 0.06. Hence, there was no overall significant difference in the mean percentage or mean number of follicles per cortical strip between treatment groups in the growing and advanced follicle developmental stage categories. This would seem to indicate that primordial follicles being recruited from the primordial pool are undergoing follicular atresia.

3.1.6.2 Oocyte health

Three days of culture



Six days of culture

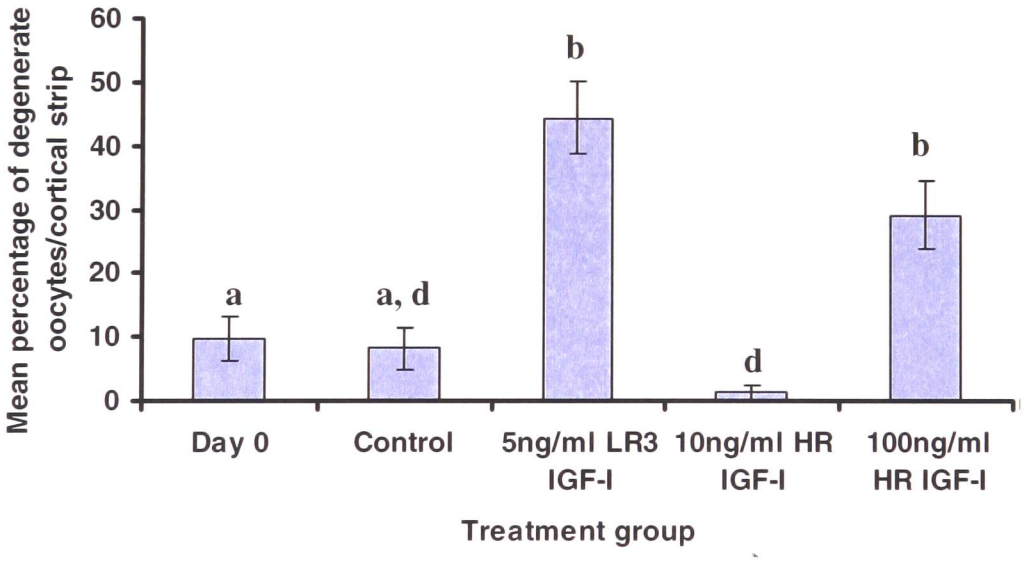


Figure 3.1.4

Effect of IGF-I on oocyte health after 3 and 6 days of culture

Oocyte health of follicles in pieces of bovine ovarian cortex after 3 and 6 days of culture.

Values are the mean percentage of degenerate oocytes per cortical strip per treatment group ± SEM for 4 cultures (3 days) and 6 cultures (6 days). Day 0, n=12, Control, n= 6, 5ng/ml LR3 IGF-I, n=6, 10ng/ml HR IGF-I, n=6 and 100ng/ml HR IGF-I, n=6.

No significant differences were detected in the mean percentage of oocyte degeneration between any of the treatment groups after three days of culture. After six days, the level of oocyte degeneration was significantly lower in control and 10ng/ml HR IGF-I treatment groups, but higher in the presence of 5ng/ml LR3 IGF-I ($p<0.05$) or 100ng/ml HR IGF-I ($p<0.05$) (Figure 3.1.4).

3.1.7 Discussion

Regulation of early follicle development is less well understood than the later stages. It has been highlighted that IGF-I is of critical importance during follicular development as the follicles of *Igf1* null mice arrest at the late preantral/early antral stage and fail to respond to gonadotrophins (Baker *et al.* 1996; Zhou *et al.* 1997). This result implies that IGF-I is not required until later stages of follicular development. However, the identification of the presence of the type I IGF receptor from the preantral stage in bovine follicles (Armstrong *et al.* 2002) and the primordial stages in human follicles (Qu *et al.* 2000) suggests the possibility of IGF playing a role in earlier stages of follicle growth.

The effects of IGFs have been shown to be dependent on the presence or absence of IGFBPs (Jones and Clemmons 1995); when an IGF is bound to an IGFBP it is inhibited from exerting its actions. Cortical strip cultures were set up to investigate the effects of IGF-I on early follicle development over time, and when the bioavailability of IGF was and was not regulated by the IGFBPs. This present study confirms the results of others studies (Braw-Tal and Yossefi 1997; Derrar *et al.* 2000; Wandji *et al.* 1996), that the culture of bovine cortical slices causes massive primordial follicle initiation, as shown by the significant decrease in primordial follicles present after culture. Furthermore, it can be seen that the final outcome of the present study is not affected by the method of analysis, as both analyses of the mean percentage and the mean number of follicles per cortical strip resulted in the same conclusions. However, histological examination of the uncultured strips showed that the mean percentage (23.5 ± 2.6 %) and mean number (6 ± 1.5) of primordial follicles per cortical strip were much lower than those observed in previous work on bovine cortical strips, namely 70.1 ± 3.5 % primordial follicles and 21 ± 5 primordial follicles per section (Derrar *et al.* 2000; Wandji *et al.* 1996). This discrepancy in values could be due to the fact that material used in the current study to look at primordial follicle activation came from mature heifers compared to the fetal tissue used in other studies (Derrar *et al.* 2000; Wandji *et al.* 1996). A greater total number of primordial follicles would be expected to be present in the fetal material when compared to the material taken from the ovaries of reproductively

mature cattle. In addition, in the present study it was observed that unlike in previous studies on bovine cortical strips (Derrar *et al.* 2000; Wandji *et al.* 1996) no significant increase in primary follicles was found after culture. Derrar *et al.* (2000) and Wandji *et al.* (1996) surprisingly found a dramatic increase in the number of primary follicles after only two days of culture. These results show an incredible acceleration in follicle growth, as the development of a primordial follicle to the early stages of growing *in vivo* is a protracted process. The failure to detect an increase in growing follicles after three and six days of culture in the present study suggests that the primordial follicles present were undergoing atresia and therefore not developing on to the primary stage. On the other hand, these results may indicate that, unlike in the fetal material, not enough follicles were present to allow the dramatic increase in follicles reaching the primary developmental stage to be seen. Thus, if primordial follicle numbers are truly as low as observed in the data presented here then these results may imply that the primordial follicle supply could run out sooner than expected and so support the notion that follicular renewal may occur in the postnatal mammalian ovary (Johnson *et al.* 2004).

Previous work has shown that when human ovarian cortex was cultured in medium supplemented with IGFs there was an increase in the percentage of follicles in the primary stage (Louhio *et al.* 2000). To date, bovine primordial follicles have not been shown to have functional receptors for IGF-I, so it is not known whether they can react to this factor (Armstrong *et al.* 2002; Wandji *et al.* 1992). Furthermore, no positive effects of IGF have been shown on activation or development of activated follicles in cultured bovine cortical pieces (Derrar *et al.* 2000; Kezele *et al.* 2002b; Yang and Fortune 2002). Results from this current study are in agreement with these findings as no significant increase in follicle initiation or progression to more advanced stages of follicle development were observed in any of the treatment groups in both the three and six day cultures. The results further support the view that IGF-I is not essential until later in development (Baker *et al.* 1996), and that IGF-I has differential effects on follicle development that are dependent on the developmental stage of the growing follicle. In addition, unlike the experiments carried out by Yang and Fortune (2002) the present study failed to observe the

negative effects of IGF-I found on primordial follicle activation. However, the failure to see any effect of IGF-I could be due again to the fact that previous studies (Derrar *et al.* 2000; Yang and Fortune 2002) used fetal material while in the present study cortical strips were taken from reproductively mature heifers. The total number of primordial follicles present may therefore not have been adequate for a true effect to be seen.

Results from recent work reveal that IGF-I had a dose-dependent negative effect on follicle growth and health, with the addition of IGF-I antibody ameliorating these harmful effects (Yang and Fortune 2002). By contrast, the morphological health of primordial, primary and secondary follicles was not compromised, but was also not improved by the presence of IGF-I in studies carried out by Derrar *at el.* (2000). Primordial oocyte health was not analysed in this current study as the culture system caused wholesale activation. Therefore, after culture most strips had very few or no primordial follicles left, which meant a valid investigation on the effects of IGF-I on primordial oocyte health could not be carried out. After three days of culture, the oocyte health of growing follicles was not altered by the addition of IGF-I to the culture medium when compared to follicles cultured in the absence of IGF-I. However, after six days of culture the addition of IGF-I in a non-regulated manner (either in the form of 5ng/ml LR3 IGF-I or 100ng/ml HR IGF-I) was found to have a deleterious effect on oocyte health. From this we can infer that, as is the case for bovine preantral follicles (McCaffery *et al.* 2000), over exposure of the oocytes to IGF-I during very early stages of bovine follicle development is detrimental to further *in vitro* follicle development.

In conclusion, the present data found no effects of IGF-I on primordial follicle initiation or activated follicle growth. IGF-I that was not regulated by IGFBPs (5ng/ml LR3 IGF-I and 100ng/ml HR IGF-I) was found to have a negative effect on the oocyte health of follicles cultured for six days. This suggests that during the early stages of follicle development the regulation of the bioavailability of IGF is crucial to maintain the health of the oocyte. The fact that IGF-I and IGF-II have been shown to play a role in stimulating the progression of early stages of human follicular

development highlights the importance of identifying species-specific differences in the regulation of follicle growth that can be used to develop and improve existing *in vitro* culture systems. Furthermore, it is now clear that the intraovarian mechanisms involved in regulating follicular grow are multi-factorial with different factors exerting effects both alone and in interaction with other factors. BMP-15 has been shown to increase mRNA levels of KL in a dose-dependent manner (Thomas *et al.* 2004). Furthermore, the interaction between BMP-15 and KL has been found to play a role in regulating granulosa cell mitosis in mice (Otsuka and Shimasaki 2002). Hence, further work into not only the role of intraovarian factors alone but also how these factors interact with one another is needed to broaden our understanding of the pivotal roles of these factors during early follicle development.

3.2 The effect of the interaction between IGF-I and androgen on primordial follicle initiation and early follicle development, and detection of androgen receptor immunoreactivity in the bovine ovary

3.2.1 Introduction

The first visible signs of primordial follicle activation and early growth are the transformation of granulosa cells from a flattened appearance to a cuboidal shape. The primary follicle is made up of an oocyte surrounded by a single layer of cuboidal granulosa cells. During this stage the granulosa cells begin to proliferate and form multiple layers around the oocyte, thus increasing the follicle diameter. The factors involved in follicle initiation and early growth are still unclear. Therefore, if we are going to be able to grow follicles *in vitro* from this very early period of development to a stage where the oocyte can be matured, then it is vital that we understand more fully the processes involved in follicle activation and early growth.

Follicles must be able to respond to endocrine and paracrine signals throughout follicle growth in order to be able to further develop and avoid undergoing atresia. Follicular atresia is hormonally controlled apoptosis, which can be stimulated or inhibited by various factors (Kaipia and Hsueh 1997). Follicle-stimulating hormone (FSH) (Chun *et al.* 1996; Markstrom *et al.* 2002) and oestrogen (Billig *et al.* 1993) are two factors that have been shown to aid developing follicles in escaping atresia. FSH's major role is to increase oestrogen biosynthesis by elevating aromatase expression in granulosa cells (Kaipia and Hsueh 1997). Oestrogen is produced by the conversion of the steroidogenic precursor, androgen, into oestrogen by the enzyme P450arom (Figure 1.4) (Hillier and Tetsuka 1997). Hence, androgens are essential during the later stages of development, and their availability can have an indirect effect in follicle development. Androgens can also have a direct action via androgen receptors (AR). Receptors for androgen have been detected in follicles from mice (Cheng *et al.* 2002), rats (Hirai *et al.* 1994; Szoltys and Slomczynska 2000; Szoltys *et al.* 2003; Tetsuka *et al.* 1995; Tetsuka and Hillier 1996), pigs (Cardenas and Pope 2002; Garrett and Guthrie 1996; Slomczynska *et al.* 2001), cattle (Hampton *et al.* 2004) humans (Saunders *et al.* 2000) and primates (Duffy *et al.* 1999; Hild-Petito *et*

al. 1991; Hillier *et al.* 1997; Saunders *et al.* 2000; Weil *et al.* 1998). Intense AR immuno staining has been found in porcine granulosa cell nuclei of preantral and antral follicles, with only weak staining on the nuclei of oocytes and theca cells (Cardenas and Pope 2002). In the rat (Tetsuka and Hillier 1996) and primate (Hillier *et al.* 1997; Weil *et al.* 1998) ovary, AR is also predominantly expressed in granulosa cells, with expression being highest in preantral/early antral follicles and gradually decreasing as the follicles mature (Tetsuka and Hillier 1997). In the primate ovary, AR mRNA is expressed in theca interna and stromal cells, and in granulosa cells to a lesser degree (Weil *et al.* 1999). Specific androgen binding sites have been characterised within ovine ovarian follicles and a direct correlation between the level of AR expression and follicle diameter has been established. However, a role has not yet been elucidated (Campo *et al.* 1985). AR mRNA expression in the bovine ovary was found to be absent in primordial follicles but present in granulosa cells in some follicles with 1-1.5 layers of cuboidal granulosa cells, and present in all follicles with more than 2 layers of granulosa cells; its expression appeared to increase during early follicle development (Hampton *et al.* 2004).

Studies on the effect of androgens on follicular development have produced conflicting results, with some showing androgens stimulating development while others indicate inhibitory effects. Treatment of rats with androgen was shown to inhibit ovarian follicular development and function (Farookhi 1985), and increase apoptosis in the granulosa cells of early antral and preantral follicles. Similarly, mouse preantral follicles cultured in the presence of androstenedione were also observed to have a significant inhibition in follicular and oestradiol production (Almahbobi *et al.* 1995). Furthermore, androgen was shown to antagonise the anti-apoptotic effects of oestrogen (Billig *et al.* 1993). On the other hand, androgen has been shown to stimulate primate primordial follicle development (Vendola *et al.* 1999b), to enhance follicular growth and development in mouse antral follicles (Murray *et al.* 1998), and to promote follicular growth and survival of preantral and small antral follicles in the primate ovary (Vendola *et al.* 1998).

It is now clear that a complex system of cell to cell interactions between the oocyte, granulosa cells and surrounding stromal tissue are involved in activation, survival and early growth and development of the follicle. Furthermore, the expression and production of several ovarian growth factors have been implicated during early stages. One such factor is IGF-I, which has previously been shown to be influenced directly by androgens. Androgen treatment was found to promote primordial follicle initiation and to also result in a 3-fold increase in IGF-I mRNA and 5-fold increase in IGF-I receptor mRNA in primate primordial follicle oocytes (Vendola *et al.* 1999b). Similarly, a 3-4-fold increase in IGF-I mRNA in antral follicle granulosa, theca and interstitial compartments was induced by the culture of primate ovaries with androgens. IGF-I receptor mRNA significantly increased in theca cells, but less so in granulosa cells and interstitium in the presence of androgens (Vendola *et al.* 1999a). These results suggest that the actions of IGF-I through its own receptor may be influenced by the presence of androgens.

The aim of this present study was to use a cortical strip system to investigate the effect of androgen on its own or in the presence of IGF-I on primordial follicle initiation and early follicle development. Hence, this study investigated whether androgen could have a direct effect via its own receptors and/or by interacting with IGF-I.

3.2.2 Materials and methods

3.2.2.1 Cortical strip isolation and culture

Cortical strips were isolated from bovine ovaries and cultured individually for 3 or 6 days, as described in Chapter 2.

3.2.2.2 Treatments

Treatment groups were as described in 3.1.2.2, but androstenedione (A4) was added to all culture media at a concentration of 10^{-7} M in all treatment groups, and was obtained from Sigma Chemicals, Poole, UK.

Treatment groups and 'n' numbers were as follows ('n' number values represent the number of bovine cortical strips cultured for each treatment group for 3 and 6 day cultures respectively):

Treatment group	'n' numbers for 3 day culture	'n' numbers for 6 day culture
A Day 0	12	12
B Control + A4	6	8
C 5ng/ml LR3 IGF-I + A4	6	6
D 10ng/ml HR IGF-I + A4	6	9
E 100ng/ml HR IGF-I + A4	6	6

Table 3.2.1

Treatment groups and number of replicates

3.2.3 Follicle classification and histological assessment

Cortical strips were fixed in paraformaldehyde, processed for histology and mounted on slides at the end of the culture period, as described in Chapter 2. Samples were sectioned at 6μm, and every fifth section was analysed throughout the whole of each cortical strip. The total number of follicles present was counted on each section. To avoid double counting, only follicles with a visible nucleolus were counted. Follicles were classed by their developmental stage (see Figures 3.1.1 and 3.1.2), and oocyte health [growing (type 1a* onwards) follicles only] was analysed by grading the

oocyte of each follicle as unhealthy (a degenerate germinal vesicle and/or misshapen oocyte) or healthy (a morphologically normal oocyte with an intact germinal vesicle).

3.2.4 Detection of androgen receptor immunoreactivity in bovine follicles

Freshly dissected out pieces of bovine ovarian cortex were fixed in paraformaldehyde, embedded in paraffin wax and mounted on slides, as described in Chapter 2. Sections were dewaxed in xylene and rehydrated from ethanol to water, before being washed in PBS (2x5 minutes, Sigma). Androgen retrieval was performed by placing sections in 0.01M citrate buffer (1x10 minutes 850W followed by 1x10 minutes 600W microwave), and then standing them for 20 minutes at RT. Sections were washed in PBS (2x5 minutes), placed in 3% hydrogen peroxide for 15 minutes and washed (5 minutes, PBS). Sections were placed in normal goat serum from a Vectastain rabbit ABC-peroxidase kit (Vector Laboratories, Southgate, UK) for 30 minutes at RT, followed by incubation with primary antibody [1 hour at RT, 1:10 dilution rabbit polyclonal androgen receptor antibody (NCL-ARp)] (Novocastra Laboratories, Newcastle Upon Tyne, UK). Negative controls were incubated with non-immune rabbit serum instead of androgen receptor antibody. Sections were washed (2x5 minutes, PBS) and incubated with a biotinylated secondary antibody (Vector Labs, as kit) for 30 minutes at RT and washed (5 minutes, PBS). Localisation of antigen was performed by incubation with DAB (Vector Labs, as kit) for 5 minutes. Slides were rinsed in tap water, counterstained with haematoxylin (15 seconds), dehydrated and mounted.

3.2.5 Statistical analysis

The mean percentage of follicles per cortical strip and the mean number of follicles per cortical strip for each treatment group were calculated for the following developmental stage categories: non-growing (type 1), growing (type 1a* and 1a) and advanced (type 2 onwards). The data set was normally distributed in accordance with the Anderson-Darling test, variances were homogeneous and treatment groups were randomly assigned, therefore, a powerful parametric test could be used to analysis samples. ANOVA and t-tests were used as they are both very robust

statistical tests. Mean percentage of follicles per cortical strip and the mean number of follicles per cortical strip were compared between experimental groups using one-way ANOVA, with subsequent two-sample t-tests to allow for individual comparisons between groups.

The difference between treatment groups in the mean percentages of degenerate oocytes per strip, within the growing follicles, was compared using one-way ANOVA, with subsequent two-sample t-tests to allow for individual comparisons between groups.

To clearly investigate if the interaction between androgen and IGF-I plays a role in primordial activation and the early stages of follicular development, direct comparisons were made between corresponding treatment groups in the presence or absence of androstenedione. Mean percentages of follicles per cortical strip and the mean number of follicles per cortical strip were compared between corresponding treatment groups with or without androstenedione within each follicle developmental stage category using one-way ANOVA. Oocyte health was analysed by comparing corresponding treatment groups in the presence or absence of androstenedione, when no IGF-I was present or when the bioavailability of IGF-I was (10ng/ml HR IGF-I) and was not (5ng/ml LR3) being regulated by IGFBPs. Differences in the mean percentages of degenerate oocytes per strip, within the growing follicles, were compared using two-sample t-tests.

3.2.6 Results

3.2.6.1 Follicle development

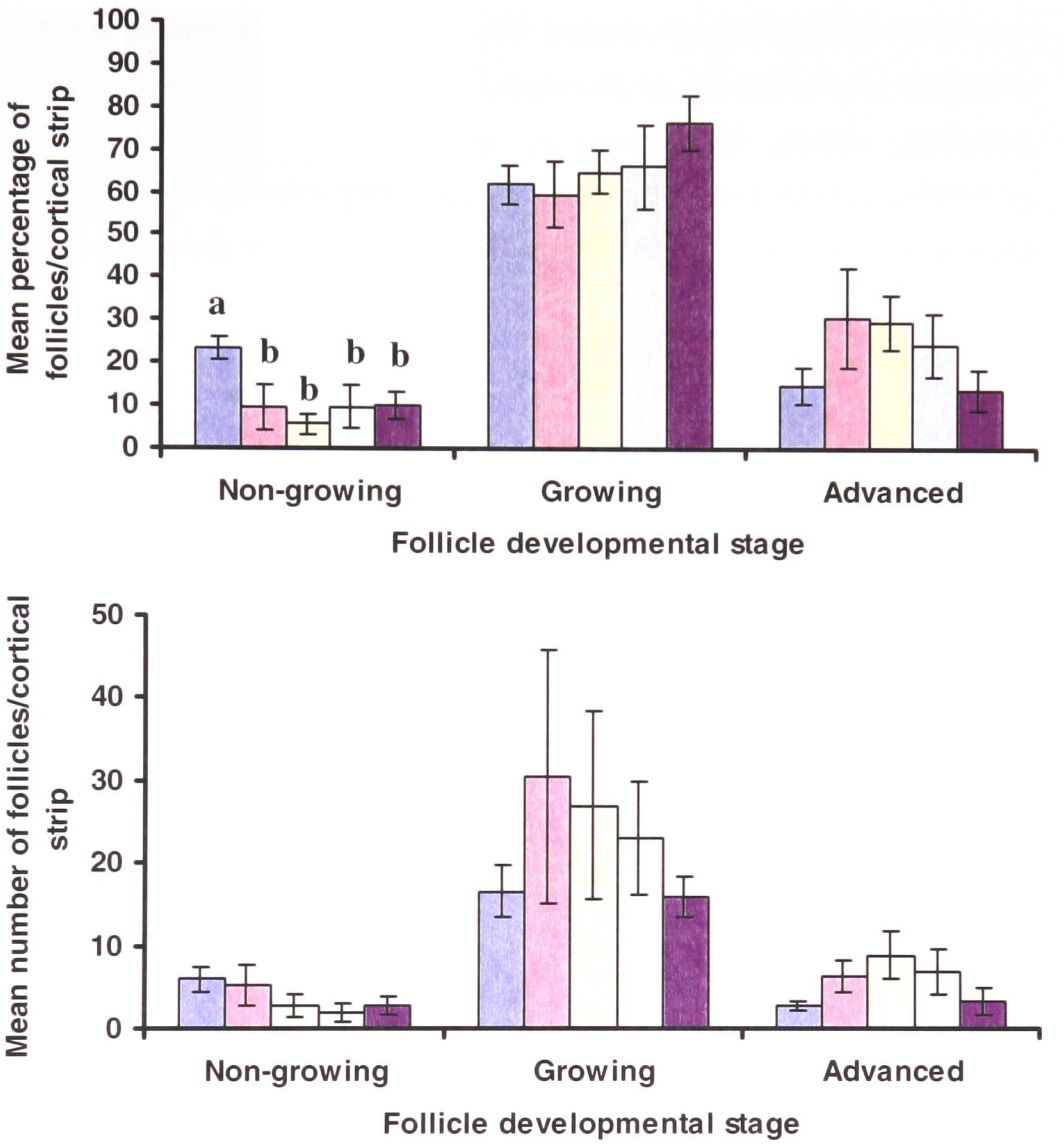


Figure 3.2.1

Effect of IGF-I in the presence of A4 on primordial follicle initiation and early follicle development after 3 days of culture

Distribution of follicles in pieces of bovine ovarian cortex after 3 days of culture.

Values are the mean percentage or number of follicles per cortical strip per treatment group ± SEM for 4 cultures.

Day 0 = (n=12), Control = (n= 6), 5ng/ml LR3 IGF-I = (n=6), 10ng/ml HR IGF-I = (n=6) and 100ng/ml HR IGF-I = (n=6).

In all treatment groups there was a significant decrease in the mean percentage of follicles per cortical strip compared to Day 0, within the non-growing developmental stage category ($p<0.05$) (Figure 3.2.1). However, there was no significant difference in the mean percentage of follicles per strip between treatment groups in the non-growing developmental stage category. Furthermore, no differences were detected in any of the follicle developmental stage categories in the growing population, between any of the treatment groups, again indicating that the primordial follicles are not growing to the later stages of development but may be being lost to follicular atresia.

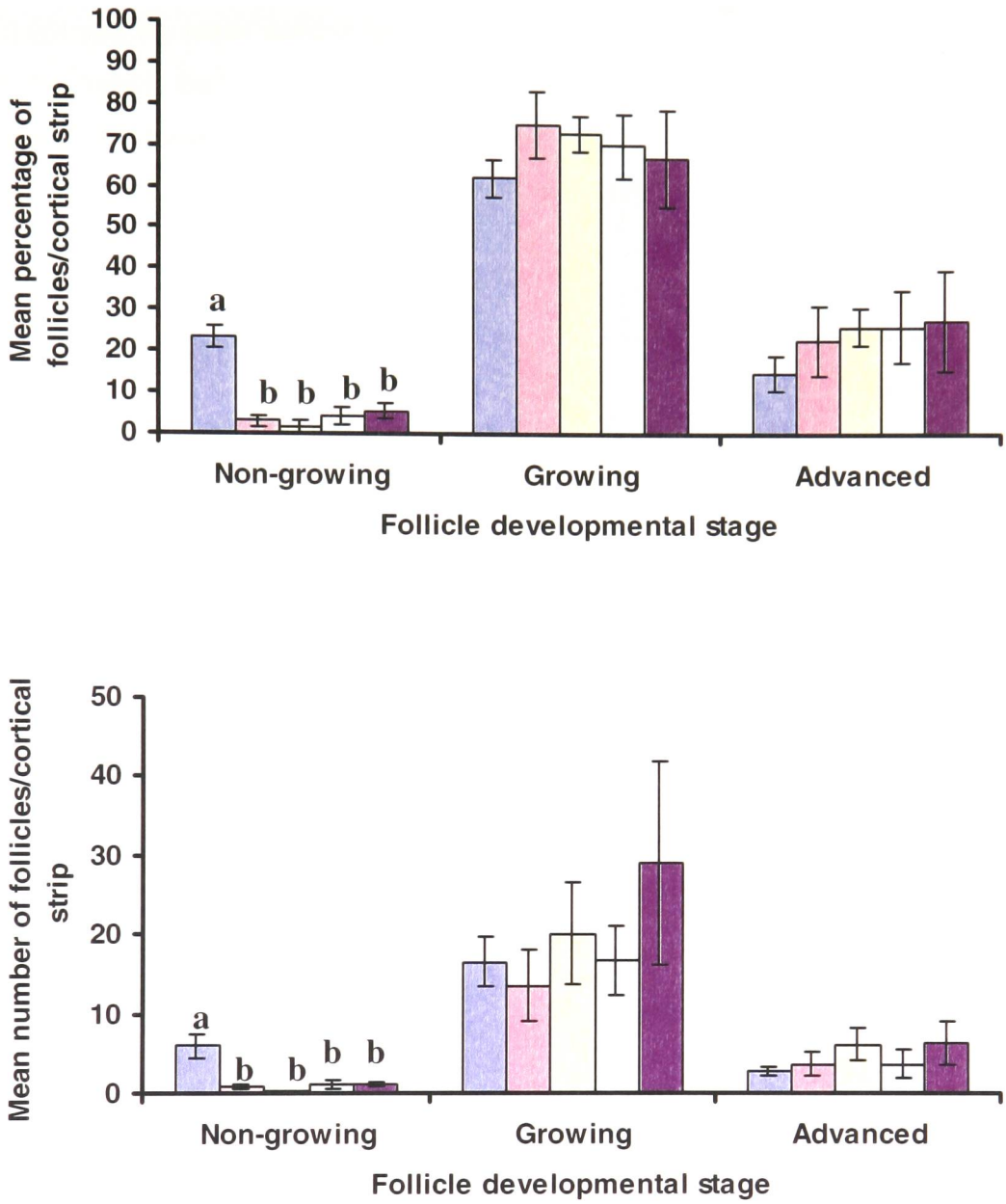


Figure 3.2.2

Effect of IGF-I in the presence of A4 on primordial follicle initiation and early follicle development after 6 days of culture

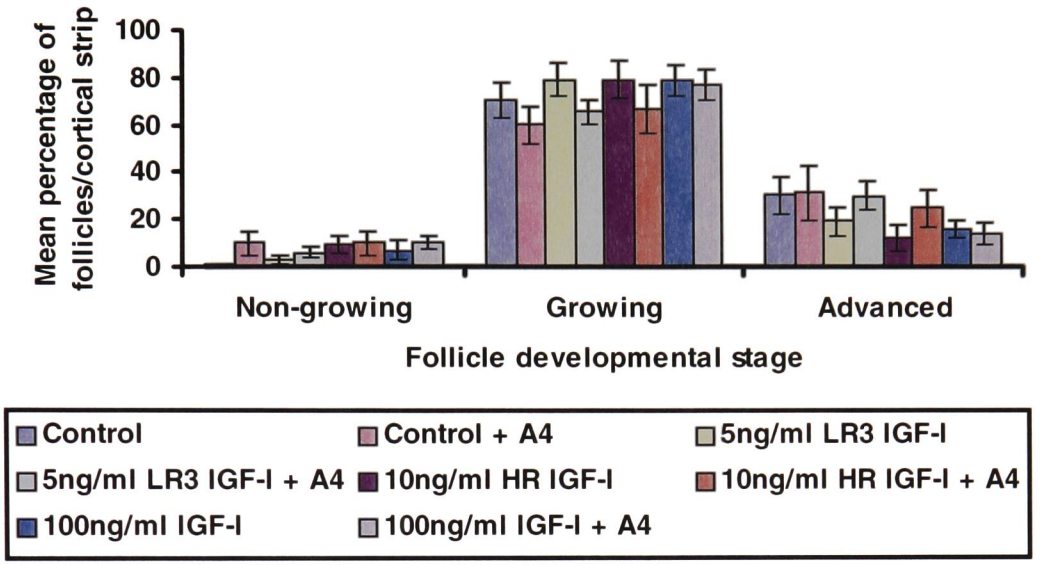
Distribution of follicles in pieces of bovine ovarian cortex after 6 days of culture.

Values are the mean percentage or number of follicles per cortical strip per treatment group ± SEM for 6 cultures.

Day 0 = (n=12), Control = (n= 8), 5ng/ml LR3 IGF-I = (n=6), 10ng/ml HR IGF-I = (n=9) and 100ng/ml HR IGF-I = (n=6).

In all treatment groups there was a significant decrease in the mean percentage ($p<0.05$) and the mean number ($p<0.01$) of follicles per cortical strip compared to Day 0, within the non-growing developmental stage category (Figure 3.2.2). However, there was no significant difference in the growing population between any of the treatment groups, suggesting that the primordial follicles are not surviving.

Three days of culture



Six days of culture

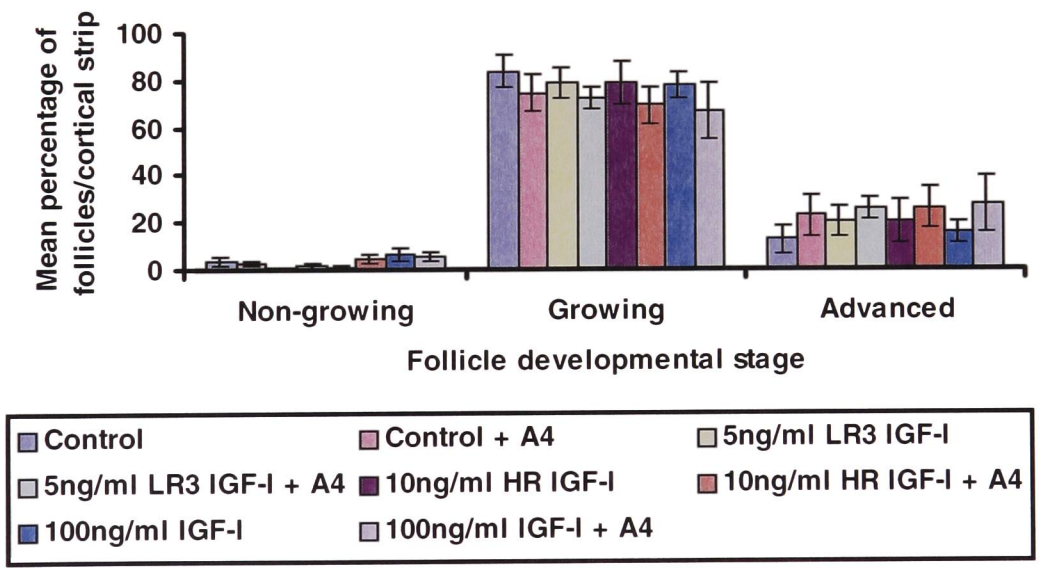


Figure 3.2.3

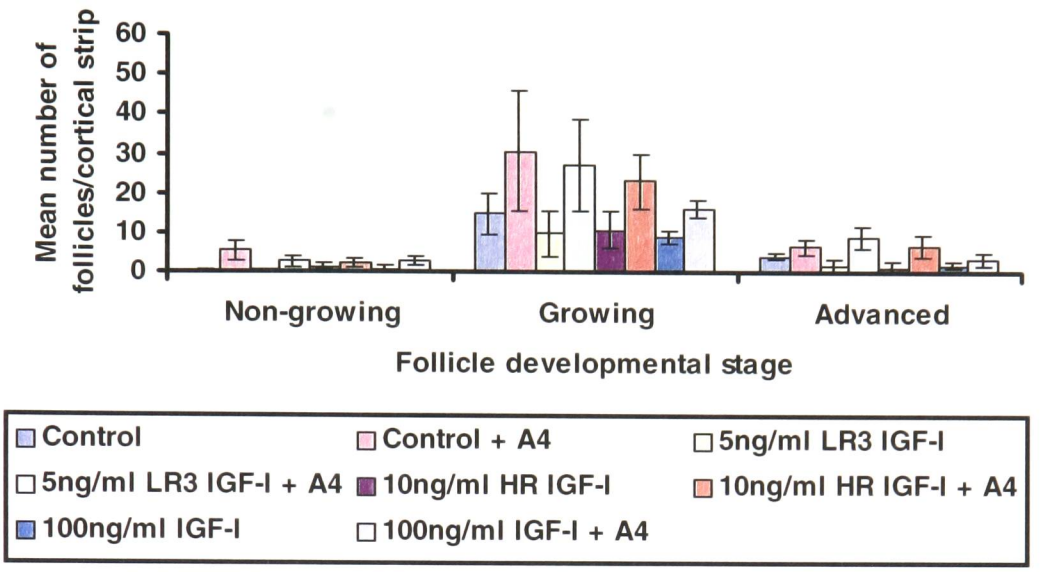
Comparison of the effects of the interaction between IGF-I and A4 on the mean percentage of follicles at different developmental stages after 3 and 6 days of culture

Distribution of follicles in pieces of bovine ovarian cortex after 3 and 6 days of culture.

Values are the mean percentage of follicles per cortical strip per treatment group ± SEM for 4 cultures (3 days) and 6 cultures (6 days). For 'n' numbers refer to Figures 3.1.2, 3.1.3, 3.2.1 and 3.2.2.

No significant difference in the mean percentage of follicles per cortical strip was found when corresponding treatment groups, with or without androstenedione within each follicle developmental stage category, were compared for three and six day cultures (Figure 3.2.3).

Three days of culture



Six days of culture

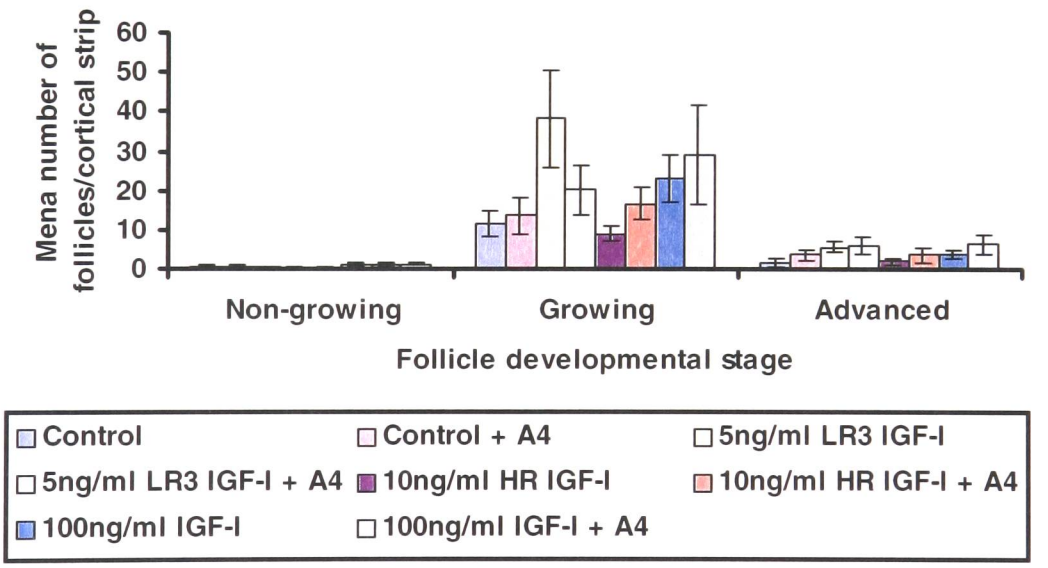


Figure 3.2.4

Comparison of the effects of the interaction between IGF-I and A4 on the mean number of follicles at different developmental stages after 3 and 6 days of culture

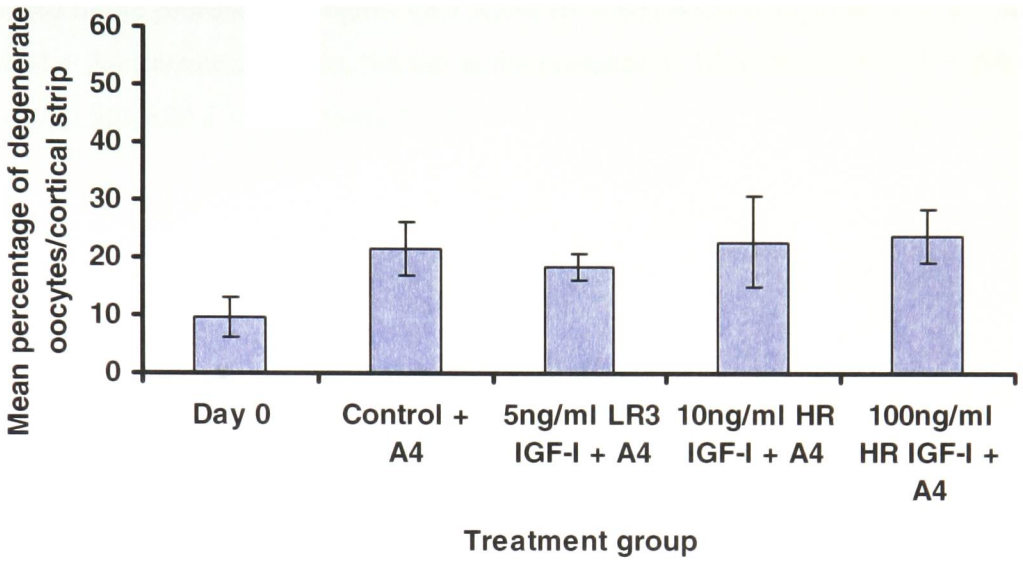
Distribution of follicles in pieces of bovine ovarian cortex after 3 and 6 days of culture.

Values are the mean percentage of follicles per cortical strip per treatment group ± SEM for 4 cultures (3 days) and 6 cultures (6 days). For 'n' numbers refer to Figures 3.1.2, 3.1.3, 3.2.1 and 3.2.2.

No significant difference in the mean number of follicles per cortical strip was found when corresponding treatment groups, with or without androstenedione within each follicle developmental stage category, were compared for three and six day cultures (Figure 3.2.4).

3.2.6.2 Oocyte health

Three days of culture



Six days of culture

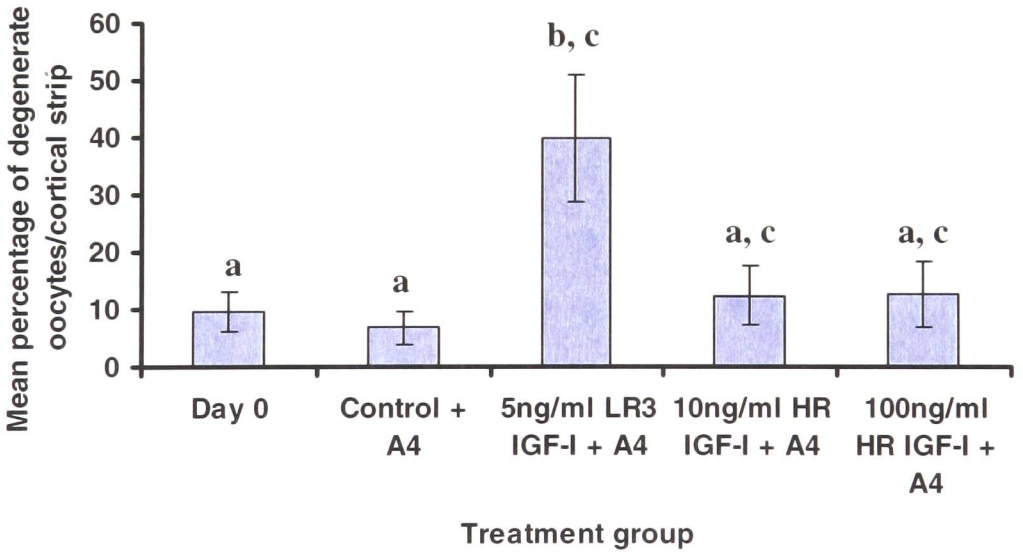


Figure 3.2.5

Effect of IGF-I in the presence of A4 on oocyte health after 3 and 6 days of culture

Oocyte health of follicles in pieces of bovine ovarian cortex after 3 and 6 days of culture.

Values are the mean percentage of degenerate oocytes per cortical strip per treatment group ± SEM for 4 cultures (3 days) and 6 cultures (6 days). Day 0, n=12,12, Control, n= 6,8, 5ng/ml LR3 IGF-I, n=6,6, 10ng/ml HR IGF-I, n=6,9 and 100ng/ml HR IGF-I, n=6,6 for 3 days and 6 days respectively.

No significant differences were detected in the mean percentage of oocyte degeneration between any of the treatment groups after three days of culture. After six days the level of oocyte degeneration was significantly higher in cortical strips cultured in the presence of 5ng/ml LR3 IGF-I ($p<0.05$) when compared to Day 0 and control + A4 treatment groups, but not in the presence of 10ng/ml HR IGF-I + A4 or 100ng/ml HR IGF-I + A4 (Figure 3.2.5).

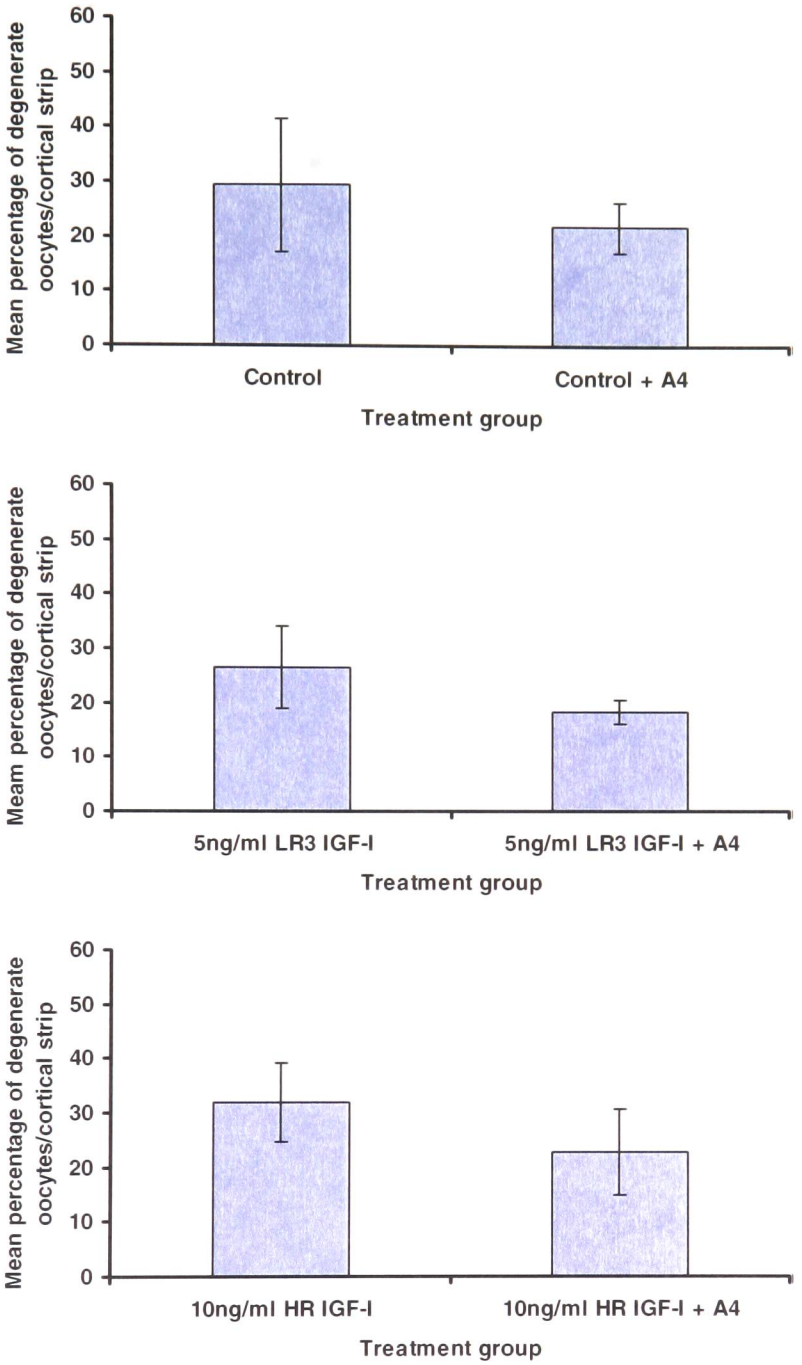


Figure 3.2.6

Oocyte health in the presence or absence of A4, when no IGF-I was present or when the bioavailability of IGF-I was and was not being regulated by IGFBPs after 3 days of culture

Values are the mean percentage of degenerate oocytes per cortical strip per treatment group ± SEM.

Values are the mean percentage of follicles per cortical strip per treatment group ± SEM for 4 cultures. For 'n' numbers refer to Figures 3.1.4 and 3.2.5.

There was no significant difference found in the mean percentage of oocyte degeneration between any of the treatment groups when corresponding treatment groups, with or without androstenedione were compared (Figure 3.2.6).

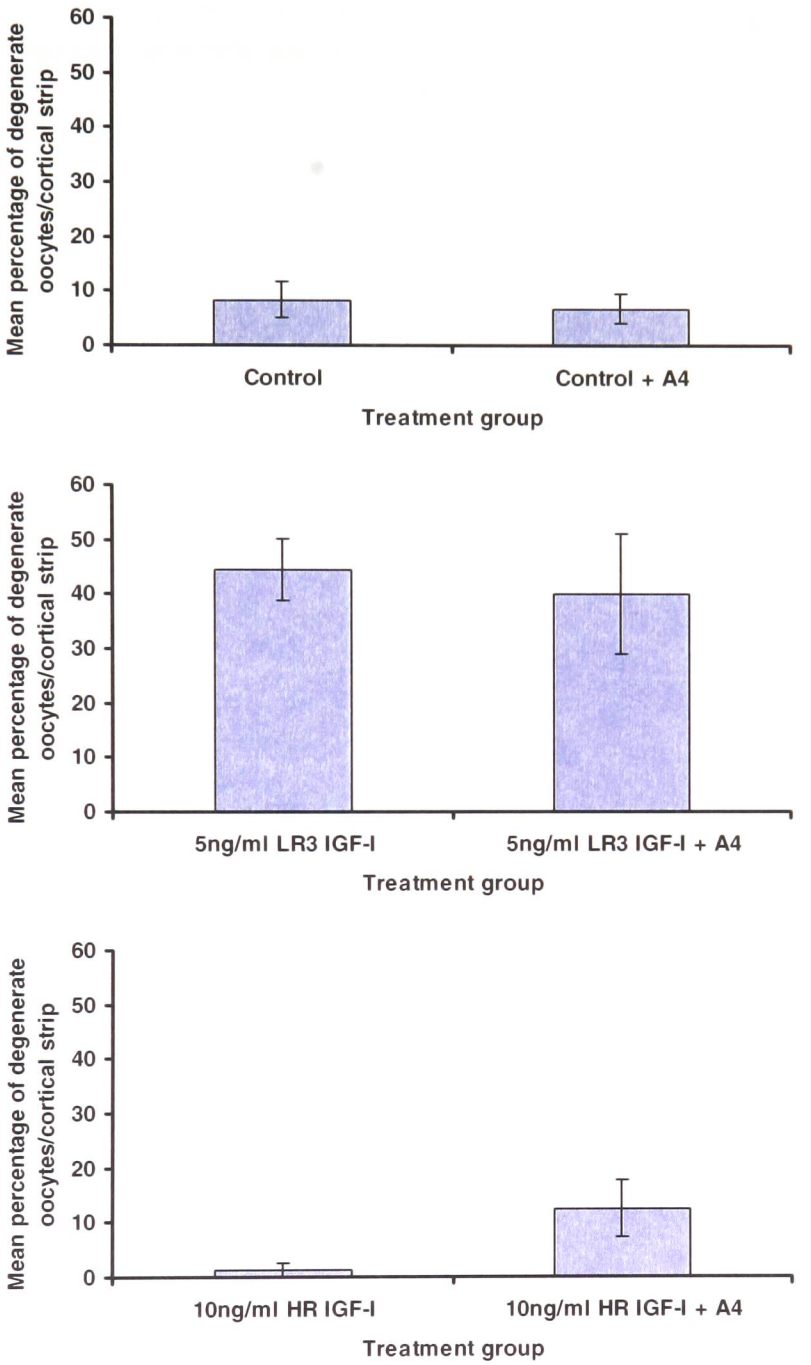


Figure 3.2.7

Oocyte health in the presence or absence of A4, when no IGF-I was present or when the bioavailability of IGF-I was and was not being regulated by IGFBPs after 6 days of culture

Values are the mean percentage of degenerate oocytes per cortical strip per treatment group ± SEM.

Values are the mean percentage of follicles per cortical strip per treatment group ± SEM for 6 cultures. For 'n' numbers refer to Figures 3.1.4 and 3.2.5.

There was no significant difference found in the mean percentage of oocyte degeneration between any of the treatment groups when corresponding treatment groups, with or without androstenedione were compared (Figure 3.2.7).

3.2.6.3 Androgen receptor immunolocalisation in the bovine ovary

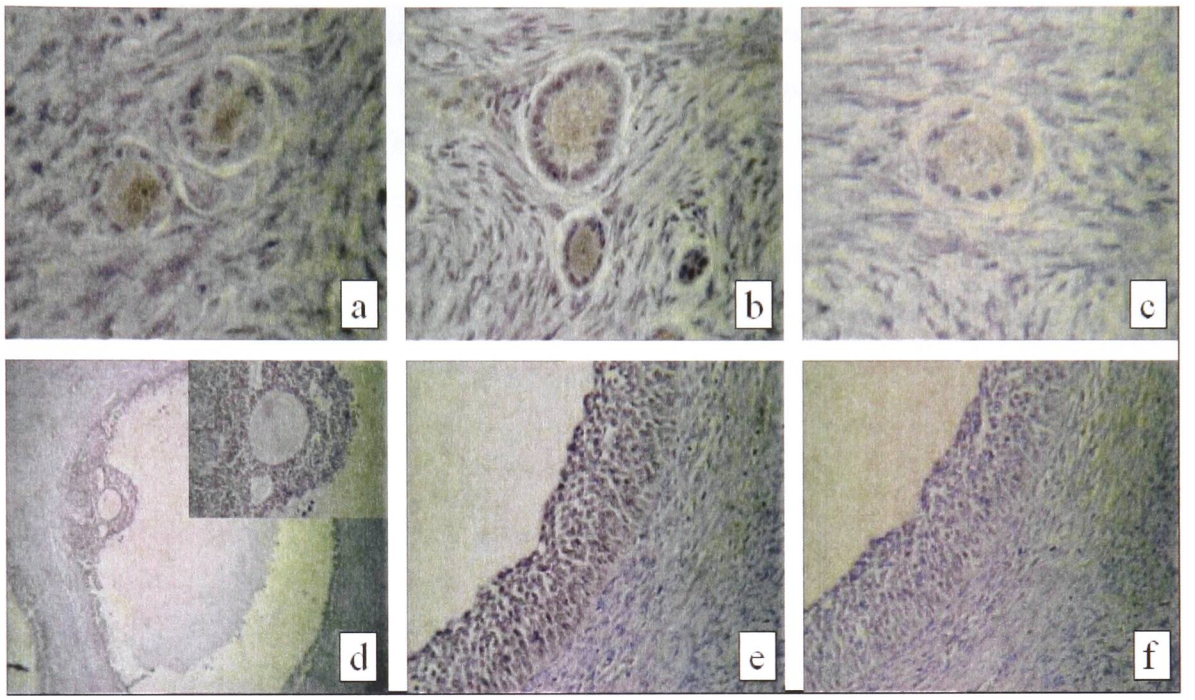


Figure 3.2.8

Androgen receptor immunoreactivity in bovine follicles

Histological sections of bovine ovarian follicles after immunocytochemistry with normal goat serum (**c** and **f**) or an antibody raised against rabbit androgen receptor (**a**, **b**, **d** and **e**).

Androgen receptor immunoreactivity was detected in granulosa cells and oocytes of preantral and antral follicles *in vivo*, with no specific staining in theca or stromal tissue (a, b, d and e). Negative control sections are represented in c and f.

3.2.7 Discussion

During the early stage of follicular development *in vitro* it is crucial to provide the growing oocyte and surrounding somatic cells with the correct sequence of factors to stimulate granulosa cell proliferation and oocyte growth, without causing inappropriate exposure of the oocyte to deleterious factors or stimulating untimely somatic cell differentiation. The culture system described in this current study was originally developed to induce differentiation of ovine and bovine granulosa cells (Campbell *et al.* 1996; Gutierrez *et al.* 1997), and was subsequently adapted for the culture of bovine preantral follicles (Gutierrez *et al.* 2000; McCaffery *et al.* 2000; Thomas *et al.* 2001). Androstenedione was included in the culture media to act as a substrate for oestradiol production.

Both androgens (Weil *et al.* 1999) and IGF-I (Spicer *et al.* 1993; Monniaux and Pisselet 1992) have been reported as promoters of the effects of FSH and hence indirectly can play a role in regulating follicular development. Furthermore, androgens and IGF-I can exert a direct action via their receptors, which have been found to be expressed in rodent (Cheng *et al.* 2002; Tetsuka *et al.* 1995; Zhao *et al.* 2002), domestic species (Armstrong *et al.* 2000; Campo *et al.* 1985; Hampton *et al.* 2004, Figure 3.2.11) and primate ovaries (el Roeiy *et al.* 1993; Horie *et al.* 1992; Weil *et al.* 1998). Androgen and IGF-I have been shown to promote primordial follicle activation (Louhio *et al.* 2000; Vendola *et al.* 1999b) and enhance follicle growth and survival (Gutierrez *et al.* 2000; Itoh *et al.* 2002; Murray *et al.* 1998; Vendola *et al.* 1998; Zhao *et al.* 2001). However, conversely the culture of fetal bovine ovarian cortex in the presence of IGF-I was found to have a dose-dependent negative effect on primordial activation and primary follicle growth (Yang and Fortune 2002). In addition, Derrar *et al.* (2000) failed to observe an effect of human recombinant IGF-I (10ng/ml) when androstenedione (10^{-7}M) was present on primordial follicle initiation, or the relative proportions of primary versus secondary follicles in bovine cortical pieces after eight days of culture. In agreement with the work carried out by Derrar *et al.* (2000), the present study found that the addition of human recombinant IGF-I and androstenedione, at the same concentrations, to the culture medium did not promote primordial, primary or secondary follicle

development. Furthermore, it was found that the addition of androstenedione with varying doses and unregulated IGF-I to the culture system also had no effect on follicle development in the non-growing and the two growing follicle developmental stages.

Recent work carried out on ovine preantral follicles found that although treatment with androstenedione increased oestradiol and testosterone production during culture, it had a deleterious effect on oocyte development (Thomas *et al.* 2002). Furthermore, IGF-I was reported recently to have a dose dependent negative effect on bovine primordial follicle activation and health (Yang and Fortune 2002) and a negative effect on the oocyte health of bovine preantral follicles (McCaffery *et al.* 2000). IGF-I in the presence of androstenedione in this current study was not found to alter the percentage of oocyte degeneration observed in cultured cortical strip treatment groups after three days of culture. However, after six days of culture the presence of 5ng/ml LR3 IGF-I significantly increased the percentage of degenerate oocytes in growing follicles when compared to cultured treatment groups containing no IGF-I or a physiological dose of IGF-I which would be regulated by IGFBPs (10ng/ml HR IGF-I). The addition of androstenedione to the culture system either on its own or in the presence of IGF-I had no effect on oocyte health.

The use of cortical strip cultures in this study has allowed the effects of IGF-I and androgen to be examined. However, as seen in other studies this culture system causes wholesale activation of follicles (Braw-Tal and Yossefi 1997; Derrar *et al.* 2000; Wandji *et al.* 1996). This activation does not appear to be normal and is probably as a result of the overstimulation by the inappropriate culture conditions. Due to this fact, future studies investigating the initiation of primordial follicles and early growth of follicles may be more informative if the technique demonstrated by Cushman et al. (2002) was used. In this study fetal bovine ovarian cortex that was grafted beneath the chorioallantoic membrane (CAM) of 6-day-old chick embryos did not exhibited the expected primordial activation and furthermore after removal from the CAM into culture primordial follicles decreased in number and primary follicles increase in number (Cushman *et al.* 2002). This model provides a useful tool

for studying factors involved in activation of primordial follicles, however, this system is not well defined and results are still influence by follicles at different stages of development present throughout the strip.

In conclusion, results obtained from the present study showed that IGF-I was not found to have any positive or negative effects on primordial follicle initiation or on the development of activated follicles. The supplementation of the culture medium with androstenedione, either on its own or in combination with IGF-I, also failed to affect follicle activation and growth. The presence of IGF-I when its bioavailability was not regulated by IGFBPs was found to have a detrimental effect on oocyte health. Moreover, this result was not altered by the supplementation of the culture medium with androstenedione, suggesting that the actions of IGF-I through its receptor are not influenced by the presence of androgens. However, it must be recognised that androstenedione is an aromatisable androgen, and would therefore have been acting as a substrate for oestradiol production in the more developed follicles present. Bearing this in mind, and the presence of androgen receptor mRNA (Hampton *et al.* 2004) and immunoreactivity found in bovine follicles in this current study (Figure 3.2.8), further work using a non-aromatisable androgen such as dihydroxytestosterone (DHT) would allow a clearer investigation into the direct action of androgen via its receptor and a possible interaction with IGF-I.

CHAPTER FOUR

The Role of the IGF System During Development of Early Bovine Antral Follicles

4.1 The effect of IGF-I on the health and morphology of bovine antral follicles *in vitro*

4.1.1 Introduction

As previously discussed, the bioavailability of IGFs can be regulated by their association with the family of insulin-like growth factor binding proteins (IGFBPs). These IGFBPs can sequester extracellular IGFs and hence reduce the level of 'free' IGFs available for specific cell surface receptors (Giudice 1992; Hwa *et al.* 1999). This, in turn, inhibits any mitogenic actions of the IGFs. The attenuation of IGF actions by the IGFBPs is controlled by complex regulatory mechanisms that vary throughout follicular development.

During the growth of a follicle, the level of expression and involvement of different components of the IGF system have been shown in the mouse ovary to change with the stage of development and the onset of atresia (Wandji *et al.* 1998). Previous *in vitro* cultures using rat preantral follicles demonstrated that follicle development was enhanced, and the morphology of the follicles maintained, when grown in the presence of IGF and FSH (Zhao *et al.* 2001). Furthermore, IGF-I has been shown to enhance steroidogenesis in the theca cells of rat antral follicles (Cara and Rosenfield 1988).

As in rodents, IGF-I has been shown to work synergistically with gonadotrophins to promote granulosa and theca cell proliferation and differentiation in domestic species such as cattle (Spicer *et al.* 1993; Stewart *et al.* 1995) and sheep (Monniaux and Pisselet 1992; Campbell *et al.* 1998). The later stages of follicle growth have been well documented as being influenced and regulated by components of the IGF system (Armstrong and Webb 1997; Lorenzo *et al.* 1994; Schams *et al.* 1999). However, unlike the later stages of follicle development, the role of the IGF system during early follicle growth is only now beginning to be understood.

Expression of IGFs and IGFBPs, and their effects, are species specific. In cattle, type I IGF receptor mRNA has been detected in the oocyte, granulosa and theca cells of

preantral and antral follicles (Armstrong *et al.* 2000; 2002), whereas IGFBP-2 mRNA has been detected in the oocyte and granulosa cells of preantral and antral bovine follicles (Armstrong *et al.* 1998; 2002, see Chapter 5 results) (Figure 1.6). Studies indicate that the expression and production of IGFBPs in the developing follicle are dependent on both the cell type and follicle size, and are regulated by IGF-I or -II and gonadotrophins (Schams *et al.* 1999). Previous *in vitro* preantral follicle culture studies have demonstrated a positive effect of IGF-I supplementation of culture medium, as the follicles were able to grow and develop into antral follicles (Gutierrez *et al.* 2000; Itoh *et al.* 2002). However, examination of preantral follicle health showed that direct access of IGF-I to bovine preantral follicles has a negative affect on oocyte size and granulosa differentiation at that particular stage of follicular growth (McCaffery *et al.* 2000). Since the health of the oocyte is fundamental, it is crucial that the oocyte is not inappropriately exposed to factors that may have a detrimental effect and thus have implications for later development.

In many biological systems, the bioavailability and IGF receptor mediated effects of IGFs are modulated by IGFBPs. In addition to IGFs exerting an action through their own receptors, under certain conditions they can also exert an action via insulin receptors (Hwa *et al.* 1999). Both IGF receptors and binding proteins compete for free IGFs. IGFBP-2 is thought to play a role in the regulation of IGF by inhibiting the interaction of IGFs with their receptors on the oocyte and/or somatic cells. To address this point, the native form of IGF-I (which does bind to IGFBPs), human recombinant IGF-I (HR IGF-I) and the synthetic analogue Long R^3 IGF-I (LR3 IGF-I) (which does not bind to IGFBPs), have been used as supplements for the culture of bovine early antral follicles. LR3 IGF-I is known to be more potent than authentic IGF-I due to its more than 1000-fold reduced affinity for IGFBPs (Francis *et al.* 1992). Therefore, by using LR3 IGF-I as a supplement in culture medium, the binding protein regulatory mechanism can be bypassed. LR3 IGF-I has been shown to have a stimulatory effect on growth and antrum formation during long-term culture of bovine preantral follicles (Gutierrez *et al.* 1997), but by contrast it has a detrimental effect on bovine preantral oocyte health (McCaffery *et al.* 2000). It

therefore appears that IGF-I may have a differential effect on the oocyte and somatic cells at certain stages of follicle growth.

The aim of the following study was to investigate further the hypothesis that the bioavailability of IGF-I is strictly regulated according to developmental stage. This was assessed by studying the effects of IGF-I on three size ranges of bovine early antral follicle development *in vitro*. Follicle growth and health were studied and comparisons were made between treatments groups with or without the modulating effects of IGFBPs.

4.1.2 Materials and methods

Three separate experiments were carried out to investigate the effects of IGF-I on early bovine antral follicles *in vitro*. Initially, a 4 day culture was set up using one dose of LR3 IGF-I and two doses of HR IGF-I. The second experiment was set up to look at the effects of a low and high dose of HR IGF-I on IGFBP-2 expression (see Chapter 5). Follicles were cultured for 2, 4 or 6 days. The results showed a significant increase in growth and oestradiol production by day 6 of culture in the follicles that were cultured in media supplemented with 1μg/ml HR IGF-I. Therefore, to identify whether this effect was brought about by binding of the IGF-I solely through the IGF receptors, or via the IGF receptors and the insulin receptors, the third experiment used follicles cultured in media supplemented with a low dose of LR3 IGF-I for 6 days, as this form of IGF-I would not be able to bind to the insulin receptor.

Therefore, the three experiments carried out were:

1) The effect of HR IGF-I and LR3 IGF-I on the health and morphology of bovine early antral follicles in a 4 day *in vitro* culture system.

2) The effect of HR IGF-I in the presence or absence of the IGFBP regulatory mechanism.

3) The effect of LR3 IGF-I on the health and morphology of bovine early antral follicles in a 6 day *in vitro* culture system.

4.1.2.1 Follicle isolation and culture

1) Early antral follicles in two size ranges (165μm-215μm and 216μm -280μm) were isolated from bovine ovaries and cultured for 4 days, as described in Chapter 2.

2) Early antral follicles in three size ranges (165μm-215μm, 216μm -280μm and 281μm -380μm) were isolated from bovine ovaries and cultured for 2, 4 or 6 days, as described in Chapter 2.

3) Early antral follicles in the size range 165μm-215μm were isolated from bovine ovaries and cultured for 6 days, as described in Chapter 2.

For each experiment, half the medium was replaced every day and the conditioned media was analysed for oestradiol production by DELFIA hormone assay, as described in Chapter 2.

4.1.2.2 Treatments

Culture medium (control) was prepared as described in 2.3.1. Human recombinant IGF-I (HR IGF-I), which does bind to IGFBPs, was purchased from Sigma Chemicals, Poole, UK. Two doses of HR IGF-I were used, a low dose of 10ng/ml and a high dose of 1μg/ml. The low dose of 10ng/ml represented a physiological dose that would be able to bind to the IGFBPs and would therefore be regulated by the level of IGFBPs present. The high dose of 1μg/ml, on the other hand, would swamp the IGFBPs present, allowing IGF-I to bypass any regulatory mechanism of the IGFBPs and hence bind freely to IGF receptors. Under these conditions, it is also possible that the IGF-I may be able to bind to the insulin receptors, as well as the IGF receptors, to exert an action. The analogue Long R^3 IGF-I (LR3 IGF-I), which does not bind to binding proteins, was purchased from Sigma Chemicals, Poole, UK. LR3 IGF-I will, therefore, bypass any IGFBP regulatory mechanism and go straight to the IGF receptors to have an effect on the target cell.

Treatment groups for follicle culture and 'n' numbers were as follows:

Experiment	Treatment groups	'n' numbers
1	**A** Control **B** 10ng/ml HR IGF-I **C** 1μg/ml HR IGF-I **D** 5ng/ml LR3 IGF-I	121 and 106 for the size ranges 165-215μm and 216-280μm respectively.
2	**A** Control **B** 10ng/ml HR IGF-I **C** 1μg/ml HR IGF-I	123, 128 and 105 for the size ranges 165-215μm, 216-280μm and 281-380μm respectively.
3	**A** Control **B** 5ng/ml LR3 IGF-I	45, 67 and 21 for the size ranges 165-215μm, 216-280μm and 281-380μm respectively.

Table 4.1.1

Treatment groups and number of replicates

Number of replica cultures were 6, 11 and 4 for experiments 1, 2 and 3 respectively.

4.1.3 Measurement of follicle growth

Follicle diameters were measured every day using a crossed micrometer (basement membrane to basement membrane) under the dissection microscope.

4.1.4 Measurement of oestradiol production

Medium collected every day was stored at –80°C and later used to detect oestradiol production. Detection of oestradiol concentrations in conditioned media was determined by DELFIA, as described in Chapter 2.

4.1.5 Histological assessment

Follicles were fixed, processed for histology and mounted on slides at the end of the culture period, as described in Chapter 2. Oocyte:follicle ratios, oocyte health and granulosa cell health were all analysed, as described in Chapter 2. Granulosa cell health analysis was not carried out for experiment 2, as follicles were not stained with haematoxylin and eosin but instead underwent immunocytochemistry for IGFBP-2 (see Chapter 5). In experiment 2 only follicles from the 6 day cultures were histologically analysed.

Treatment groups for follicle histological assessment and 'n' numbers were as follows:

Experiment	Treatment groups	'n' numbers
1	**A** Day 0 **B** Control **C** 10ng/ml HR IGF-I **D** 1μg/ml HR IGF-I **E** 5ng/ml LR3 IGF-I	136 and 130 for the size ranges 165-215μm and 216-280μm respectively.
2	**A** Day 0 **B** Control **C** 10ng/ml HR IGF-I **D** 1μg/ml HR IGF-I	48, 43 and 41 for the size ranges 165-215μm, 216-280μm and 281-380μm respectively.
3	**A** Day 0 **B** Control **C** 5ng/ml LR3 IGF-I	56, 78 and 26 for the size ranges 165-215μm, 216-280μm and 281-380μm respectively.

Table 4.1.2

Treatment groups and number of replicates

4.1.6 Statistical analysis

The data set was normally distributed in accordance with the Anderson-Darling test, variances were homogeneous and treatment groups were randomly assigned, therefore, a powerful parametric test could be used to analysis samples. ANOVA and subsequently the multiple comparison test 'Tukeys' was used after a significant ANOVA to determine where the significant difference existed among the groups. Mean follicle diameters and oestradiol production on day 4 (experiment 1) or day 6 (experiments 2 and 3) of culture were compared between experimental groups using a repeated measures ANOVA (General Linear Model), with subsequent Tukeys tests to allow for individual comparisons between groups.

Follicles fixed for histological examination on day 4 (experiment 1) or day 6 (experiments 2 and 3) were analysed for differences between treatment groups with regard to the mean oocyte:follicle ratios and mean percentage of pyknotic granulosa cells by one-way ANOVA, with subsequent two-sample t-tests to allow for individual comparisons between groups. ANOVA and t-tests were used as they are both very robust statistical tests. The difference between treatment groups with regard to the number of degenerate oocytes was assessed by a 2-proportions test. The 2-proportions test was chosen as each group was compared to one another and this method tests whether two binomial proportions are significantly different from one another and makes allowances for unbalanced data sets.

4.1.7 Results

4.1.7.1 Follicular growth

1) The effect of HR IGF-I and LR3 IGF-I on the health and morphology of bovine early antral follicles in a 4 day *in vitro* culture system.

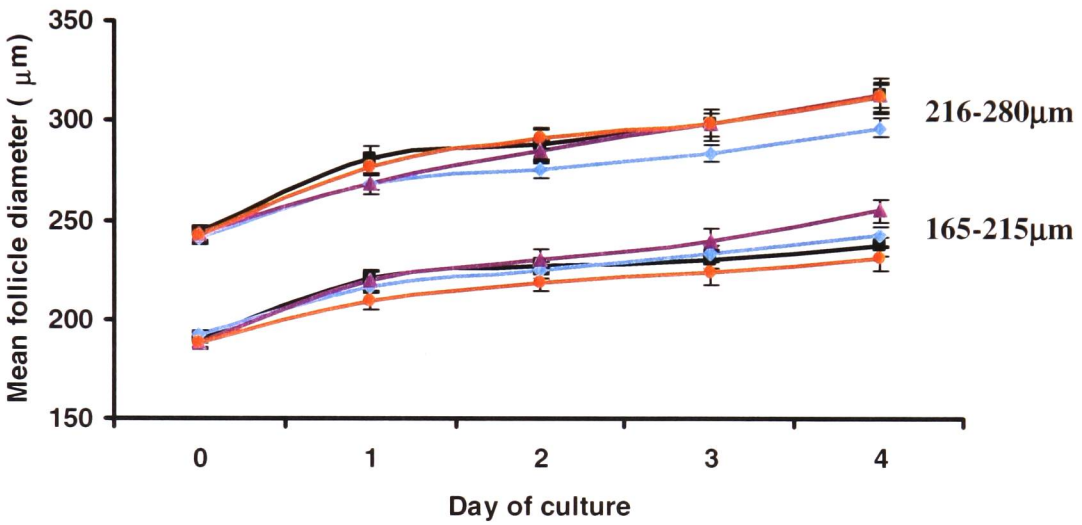


Figure 4.1.1

Effect of HR IGF-I and LR3 IGF-I on follicle growth

Growth of early antral follicles in 2 size ranges in the following treatment groups: Control = ■ (n=30,26), 10ng/ml HR IGF-I = ♦ (n=32,27), 1µg/ml HR IGF-I = ▲ (n=28,26) and 5ng/ml LR3 IGF-I = ● (n=31.27). The 'n' numbers are for 165-215µm and 216-280µm size ranges respectively. Values are mean ± SEM.

All groups showed a significant increase in size between days 0 and 4 ($p<0.01$) (Figure 4.1.1).

By day 4 there was no significant increase in follicle growth between treatment groups in the early antral follicles in all size ranges (Figure 4.1.1).

2) The effect of HR IGF-I in the presence or absence of the IGFBP regulatory mechanism.

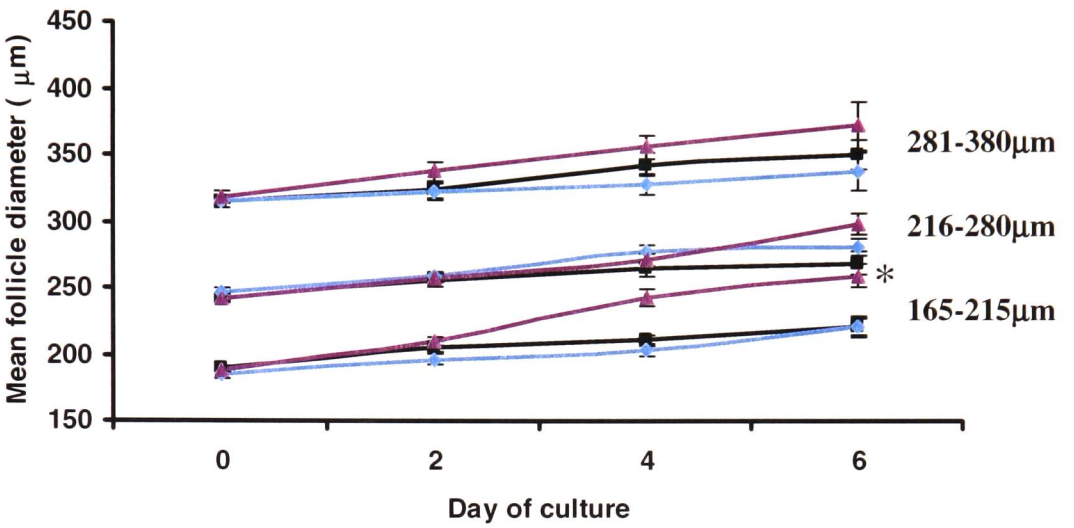


Figure 4.1.2

Effect of HR IGF-I on follicle growth in the presence or absence of the IGFBP regulatory mechanism

Growth of early antral follicles in 3 size ranges in the following treatment groups:
Control = ■ (n=43,43,35), 10ng/ml HR IGF-I = ♦ (n=37,47,35) and 1µg/ml HR IGF-I = ▲ (n=43,38,35). The 'n' numbers are for 165-215µm, 216-280µm and 281-380µm size ranges respectively. Values are mean ± SEM. * indicates a significant difference in follicle growth ($p \leq 0.05$).

All groups showed a significant increase in growth between days 0 and 6 ($p<0.01$) (Figure 4.1.2).

There was a significant increase in follicle growth by day 6 in the early antral follicles cultured in media supplemented with 1µg/ml HR IGF-I in the smallest size range (165-215µm) ($p<0.01$) (Figure 4.1.2).

Follicles in the size ranges 216-280µm and 281-380µm did not show a significant increase in follicle growth between treatment groups.

3) The effect of LR3 IGF-I on the health and morphology of bovine early antral follicles in a 6 day *in vitro* culture system.

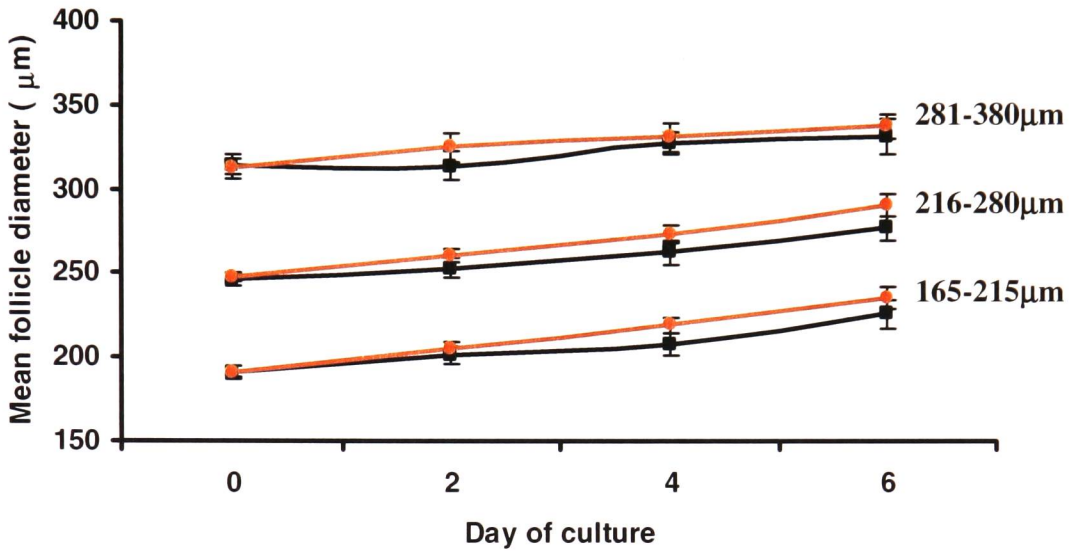


Figure 4.1.3

Effect of LR3 IGF-I on follicle growth

Growth of early antral follicles in 3 size ranges in the following treatment groups:
Control = ■ (n=18,27,8), 5ng/ml LR3 IGF-I = • (n=27,40,13). The 'n' numbers are for 165-215µm, 216-280µm and 281-380µm size ranges respectively. Values are mean ± SEM.

All groups showed a significant increase in growth between days 0 and 6 ($p<0.01$) (Figure 4.1.3).

Follicles in all size ranges did not show a significant increase in follicle growth between treatment groups (Figure 4.1.3).

4.1.7.2 Oestradiol production

1) The effect of HR IGF-I and LR3 IGF-I on the health and morphology of bovine early antral follicles in a 4 day *in vitro* culture system.

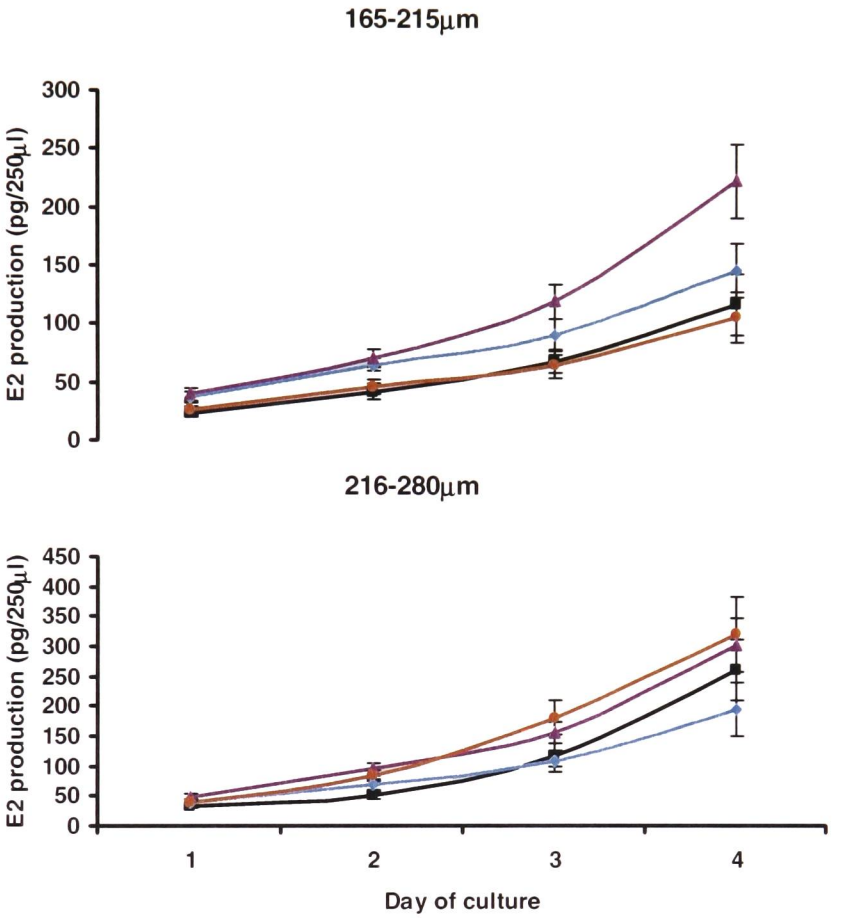


Figure 4.1.4

Effect of HR IGF-I and LR3 IGF-I on oestradiol production

Oestradiol production of early antral follicles in 2 size ranges in the following treatment groups: Control = ■ (n=30,26), 10ng/ml HR IGF-I = ♦ (n=32,27), 1µg/ml HR IGF-I = ▲ (n=28,26) and 5ng/ml LR3 IGF-I = ● (n=31.27). The 'n' numbers are for 165-215µm and 216-280µm size ranges respectively. Values are mean ± SEM.

All groups showed a significant increase in oestradiol production between days 1 and 4 ($p<0.01$) (Figure 4.1.4).

No significant difference in oestradiol production was shown between day 1 and day 4 in any treatment group across all size ranges (Figure 4.1.4).

2) The effect of HR IGF-I in the presence or absence of the IGFBP regulatory mechanism.

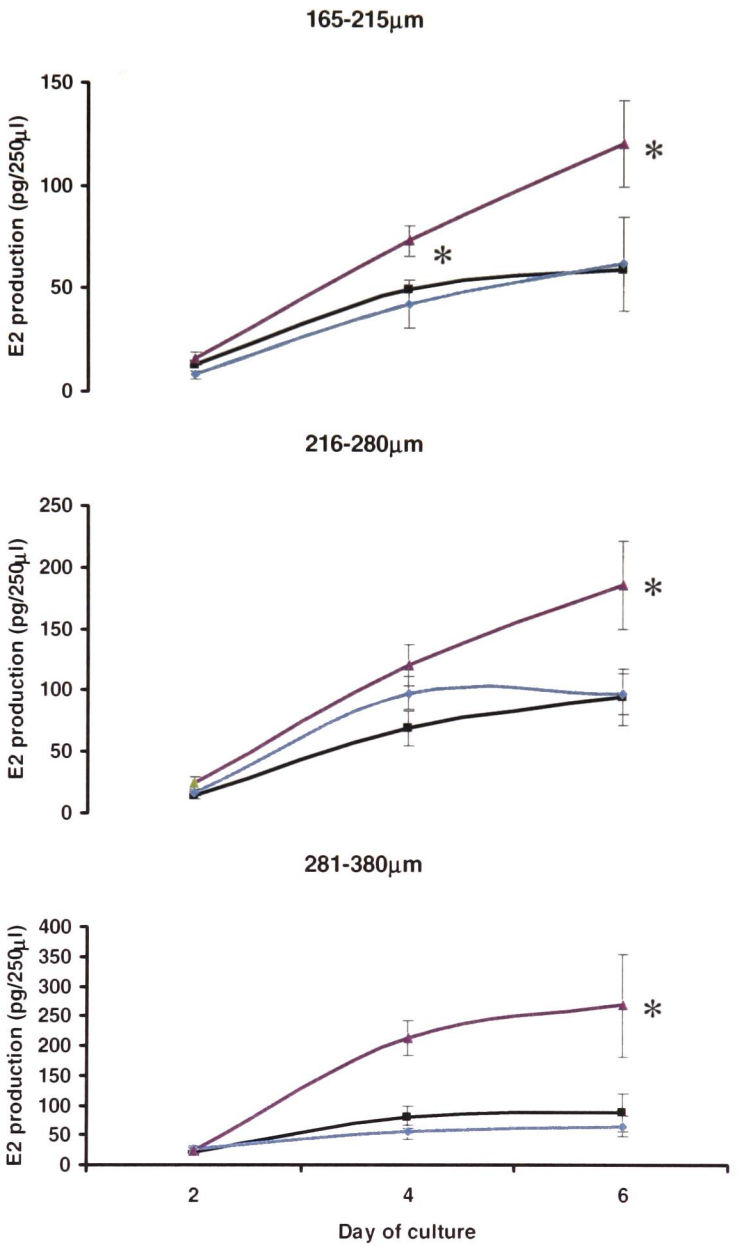


Figure 4.1.5

Effect of HR IGF-I on oestradiol production in the presence or absence of the IGFBP regulatory mechanism

Growth of early antral follicles in 3 size ranges in the following treatment groups:

Control = ■ (n=43,43,35), 10ng/ml HR IGF-I = ♦ (n=37,47,35) and 1µg/ml HR IGF-I = ▲ (n=43,38,35). The 'n' numbers are for 165-215µm, 216-280µm and 281-380µm size ranges respectively. Values are mean ± SEM. * indicates a significant difference in oestradiol production ($p \leq 0.05$).

All groups showed a significant increase in oestradiol production between days 2 and 6 ($p<0.01$) (Figure 4.1.5).

A significant increase in oestradiol production was shown between day 2 and day 6 in early antral follicles cultured in the presence of 1μg/ml HR IGF-I, across all size ranges [165-215μm ($p<0.05$), 216-280μm and 281-380μm ($p<0.01$)] (Figure 4.1.5).

3) The effect of LR3 IGF-I on the health and morphology of bovine early antral follicles in a 6 day *in vitro* culture system.

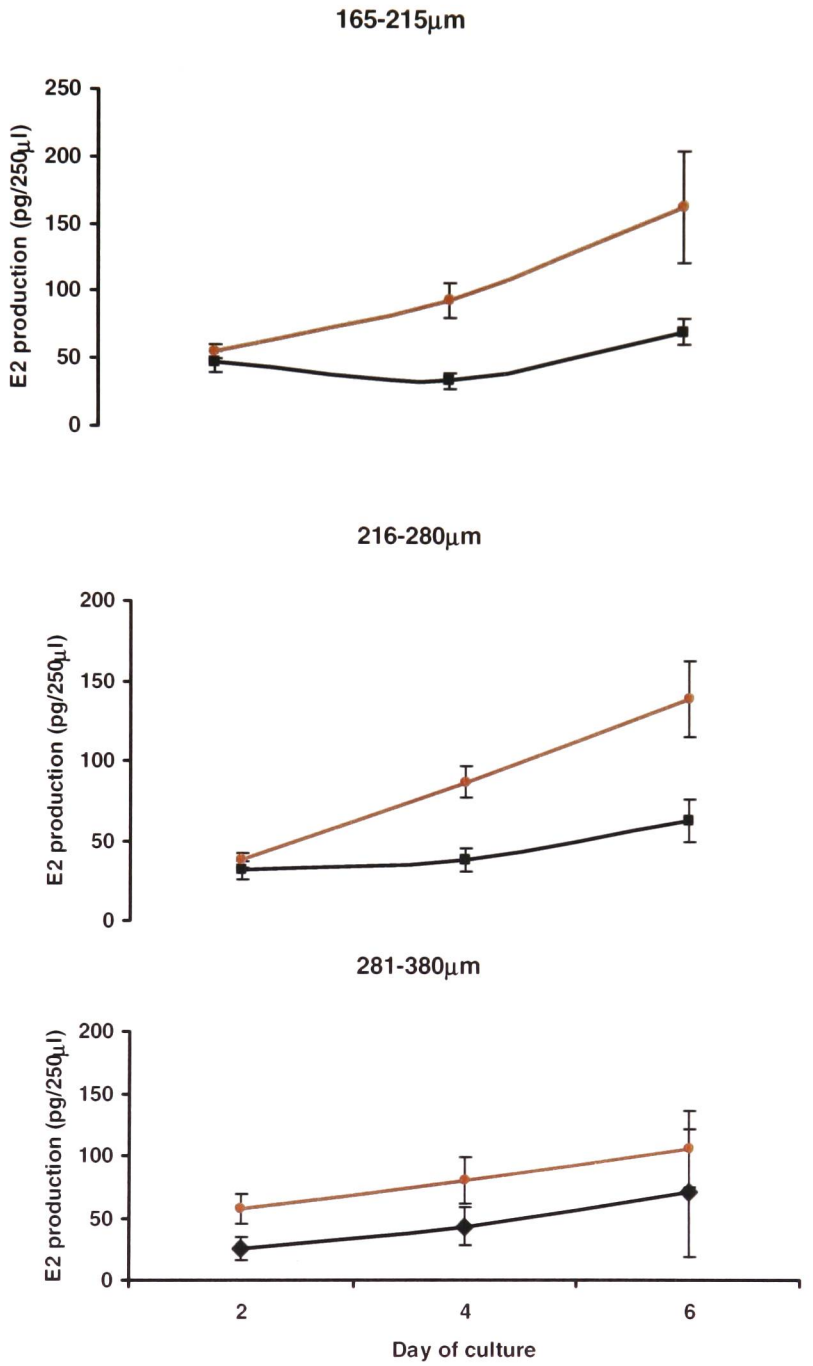


Figure 4.1.6

Effect of LR3 IGF-I on oestradiol production

Oestradiol production of early antral follicles in 3 size ranges in the following treatment groups: Control = ■ (n=18,27,8), 5ng/ml LR3 IGF-I = ● (n=27,40,13). The 'n' numbers are for 165-215µm, 216-280µm and 281-380µm size ranges respectively. Values are mean ± SEM.

Follicles in the size ranges 165-215μm and 216-280μm showed a significant increase in oestradiol production between days 0 and 6 ($p<0.01$). The largest size range (281-380μm) did not show a significant increase in oestradiol production between days 0 and 6 (Figure 4.1.6).

Follicles in all size ranges did not show a significant difference in oestradiol production between treatment groups (Figure 4.1.6).

4.1.7.3 Histological assessment

4.1.7.3.1 Oocyte health

1) The effect of HR IGF-I and LR3 IGF-I on the health and morphology of bovine early antral follicles in a 4 day *in vitro* culture system.

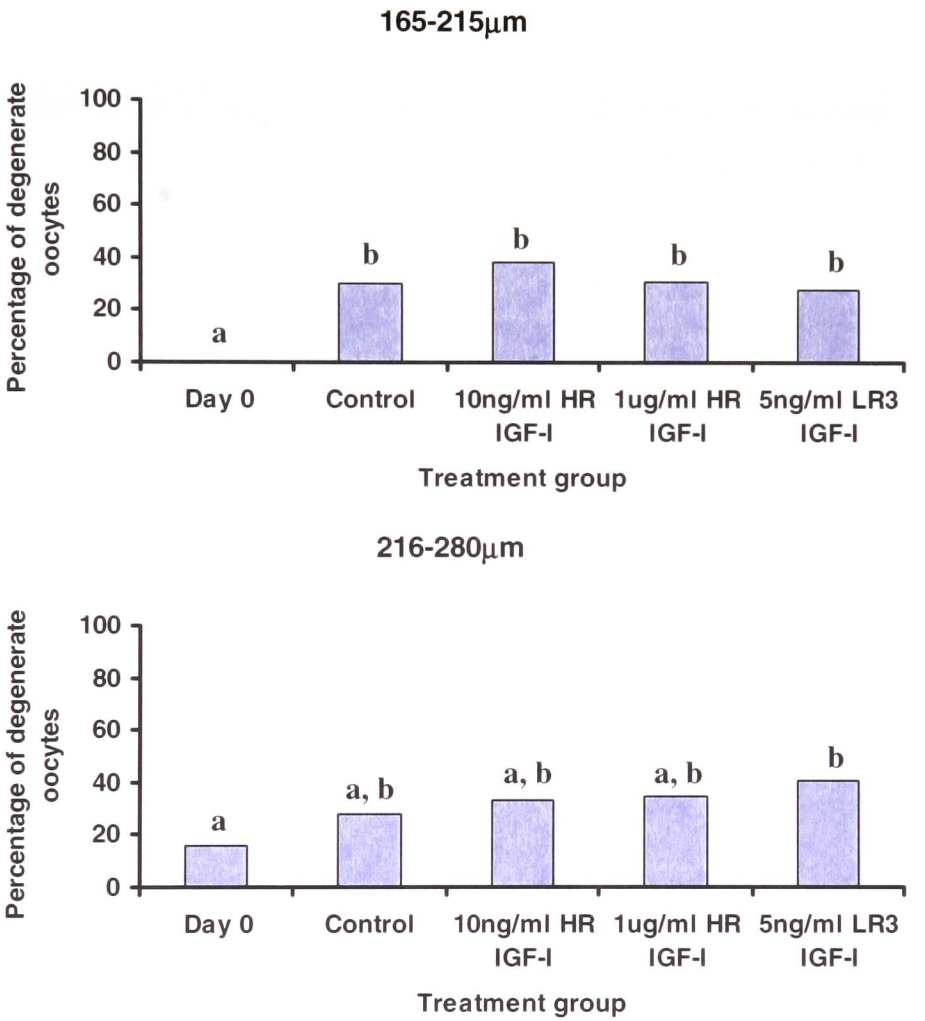


Figure 4.1.7

Effect of HR IGF-I and LR3 IGF-I on oocyte health

Oocyte health of 2 size ranges of early antral follicles in the following treatment groups: Day 0 (n=20,25), Control (n=30,25), 10ng/ml HR IGF-I (n=31,27), 1µg/ml HR IGF-I (n=26,26) and 5ng/ml LR3 IGF-I (n=29,27). Values are the number of degenerate oocytes displayed as a percentage of the total oocytes analysed after 4 days of culture. The 'n' numbers are for 165-215µm and 216-280µm size ranges respectively.

All cultured follicles in the smallest size range (165-215μm) were found to have a significantly higher percentage of degenerate oocytes compared to Day 0 follicles ($p<0.01$) (Figure 4.1.7).

There was no significant difference in the percentage of oocyte degeneration between cultured treatment groups in the size range 165-215μm (Figure 4.1.7).

In the larger size range of follicles (216-280μm) a significant increase in the percentage of degenerate oocytes was only found in follicles cultured in media supplemented with 5ng/ml LR3 IGF-I compared to the Day 0 treatment group ($p<0.05$) (Figure 4.1.7).

There was no significant difference in the percentage of oocyte degeneration between cultured treatment groups in the size range 216-280μm (Figure 4.1.7).

2) The effect of HR IGF-I in the presence or absence of the IGFBP regulatory mechanism.

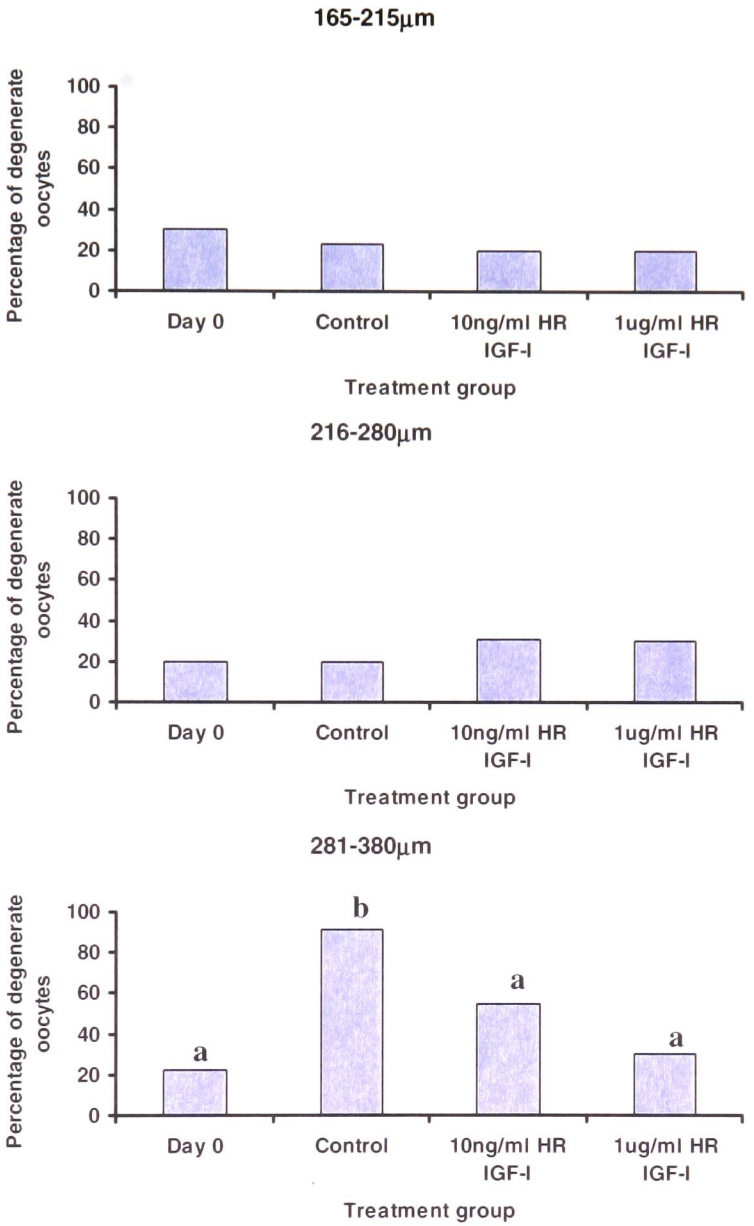


Figure 4.1.8

Effect of HR IGF-I on oocyte health in the presence or absence of the IGFBP regulatory mechanism

Oocyte health of 3 size ranges of early antral follicles in the following treatment groups: Day 0 (n=10,10,9), Control (n=13,10,11), 10ng/ml HR IGF-I (n=10,13,11) and 1μg/ml HR IGF-I (n=15,10,10). Values are the number of degenerate oocytes displayed as a percentage of the total oocytes analysed after 6 days of culture. The 'n' numbers are for 165-215μm, 216-280μm and 281-380μm size ranges respectively.

There was no significant difference in the percentage of oocyte degeneration between treatment groups in the size ranges 165-215μm and 281-380μm (Figure 4.1.8).

In the larger size range of follicles (281-380μm) a significant increase in the percentage of degenerate oocytes was found in follicles cultured in control media compared to the Day 0 ($p<0.01$), 10ng/ml HR IGF-I ($p<0.01$) and 1μg/ml HR IGF-I ($p<0.05$) treatment groups (Figure 4.1.8).

3) The effect of LR3 IGF-I on the health and morphology of bovine early antral follicles in a 6 day *in vitro* culture system.

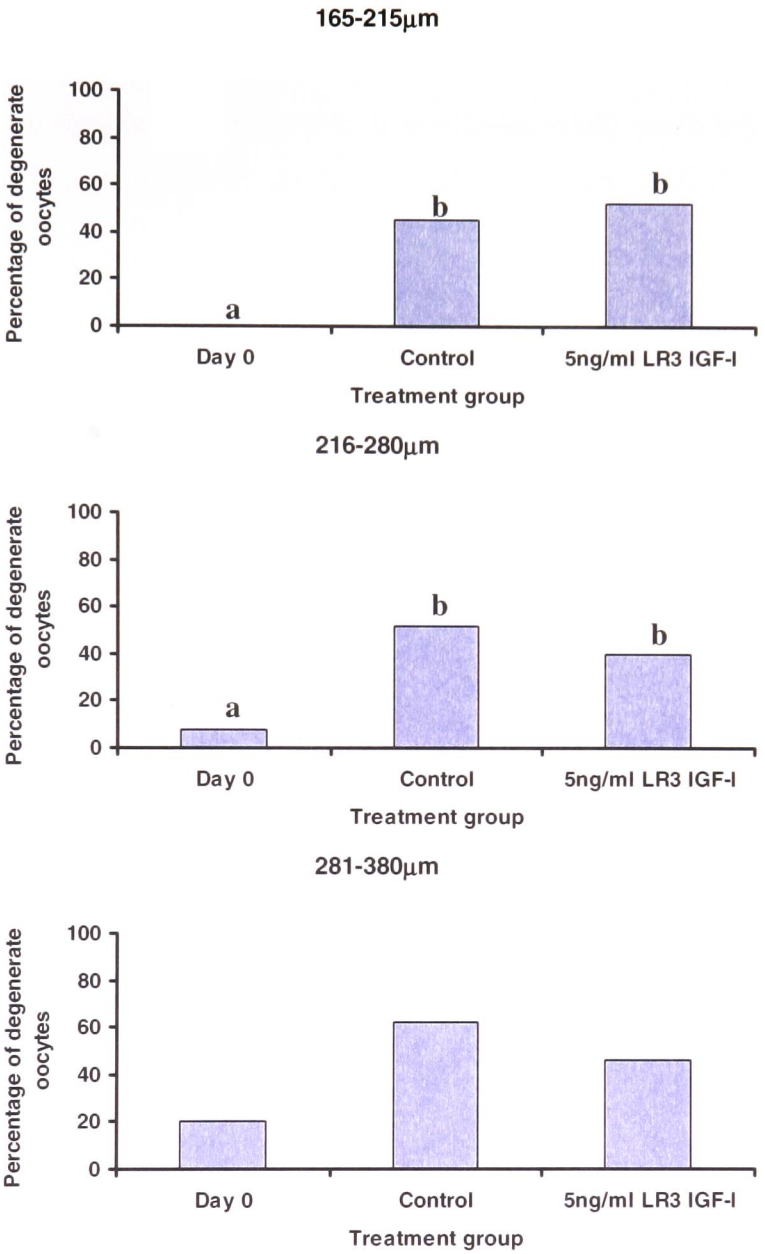


Figure 4.1.9

Effect of LR3 IGF-I on oocyte health

Oocyte health of 3 size ranges of early antral follicles in the following treatment groups: Day 0 (n=13,13,5) Control (n=18,25,8) and 5ng/ml LR3 IGF-I (25,40,13). Values are the number of degenerate oocytes displayed as a percentage of the total oocytes analysed after 6 days of culture. The 'n' numbers are for 165-215µm, 216-280µm and 281-380µm size ranges respectively.

All cultured follicles in the size ranges 165-215μm and 216-280μm were found to have a significantly higher percentage of degenerate oocytes compared to Day 0 follicles ($p<0.01$) (Figure 4.1.9).

There was no significant difference in the percentage of oocyte degeneration between cultured treatment groups in the size ranges 165-215μm and 216-280μm (Figure 4.1.9).

In the larger size range of follicles (216-280μm) no significant difference in the percentage of degenerate oocytes was found between any treatment groups (Figure 4.1.9).

4.1.7.3.2 Oocyte:follicle ratio

1/ The effect of HR IGF-I and LR3 IGF-I on the health and morphology of bovine early antral follicles in a 4 day *in vitro* culture system.

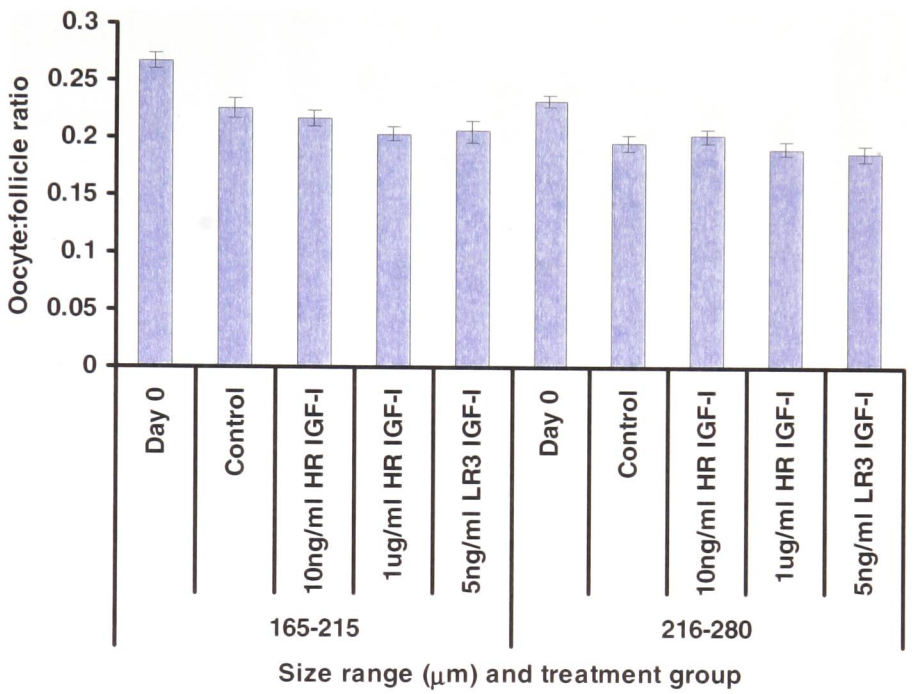


Figure 4.1.10

Effect of HR IGF-I and LR3 IGF-I on oocyte:follicle ratios

Oocyte:follicle ratios of 2 size ranges of early antral follicles in the following treatment groups: Day 0 (n=20,25), Control (n=30,25), 10ng/ml HR IGF-I (n=31,27), 1μg/ml HR IGF-I (n=26,26) and 5ng/ml LR3 IGF-I (n=29,27). The 'n' numbers are for 165-215μm and 216-280μm size ranges respectively. Values are the mean oocyte:follicle ratio per treatment group, within each size range, analysed after 4 days of culture.

No significant difference in oocyte:follicle ratio was found between treatment groups in either of the size ranges (Figure 4.1.10).

2/ The effect of HR IGF-I in the presence or absence of the IGFBP regulatory mechanism.

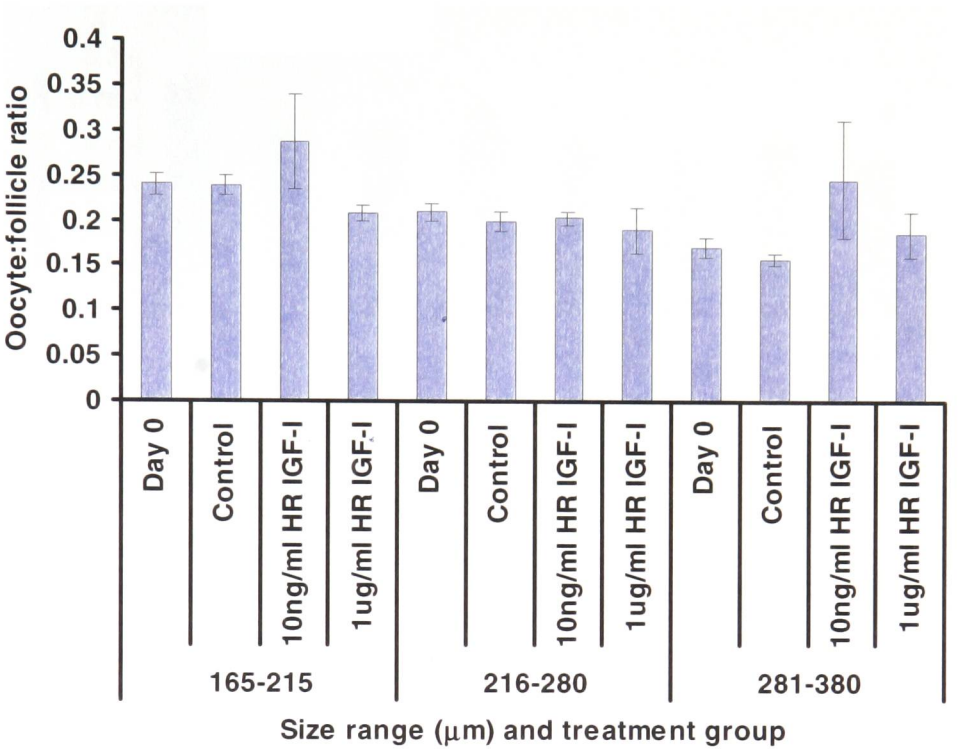


Figure 4.1.11

Effect of HR IGF-I on oocyte:follicle ratios in the presence or absence of the IGFBP regulatory mechanism

Oocyte:follicle ratios of 3 size ranges of early antral follicles in the following treatment groups: Day 0 (n=10,10,9), Control (n=13,10,11), 10ng/ml HR IGF-I (n=10,13,11) and 1μg/ml HR IGF-I (n=15,10,10). The 'n' numbers are for 165-215μm, 216-280μm and 281-380μm size ranges respectively. Values are the mean oocyte:follicle ratio per treatment group, within each size range, analysed after 6 days of culture.

No significant difference in oocyte:follicle ratio was found between treatment groups in any of the size ranges (Figure 4.1.11).

3/ The effect of LR3 IGF-I on the health and morphology of bovine early antral follicles in a 6 day *in vitro* culture system.

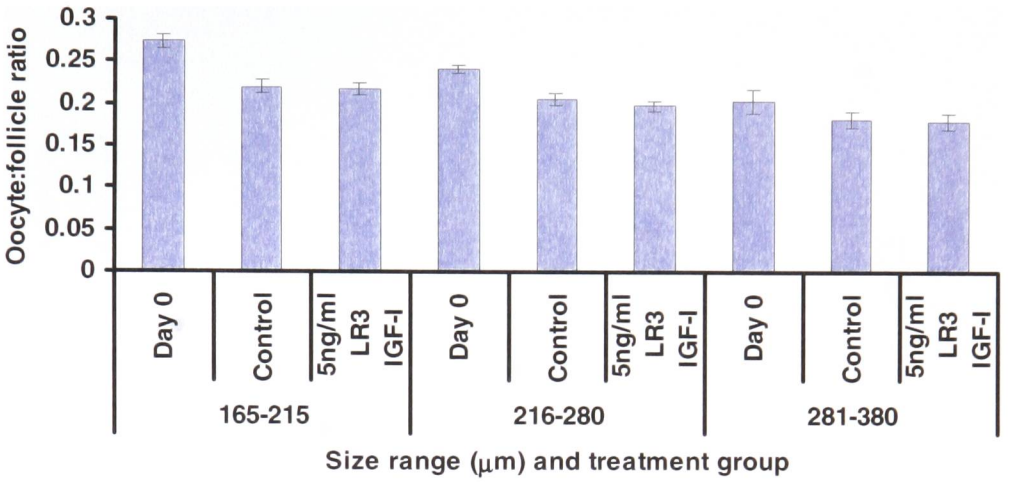


Figure 4.1.12

Effect of LR3 IGF-I on oocyte:follicle ratios

Oocyte:follicle ratios of 3 size ranges of early antral follicles in the following treatment groups: Day 0 (n=13,13,5) Control (n=18,25,8) and 5ng/ml LR3 IGF-I (25,40,13). The 'n' numbers are for 165-215μm, 216-280μm and 281-380μm size ranges respectively.

Values are the mean oocyte:follicle ratio per treatment group, within each size range, analysed after 6 days of culture.

No significant difference in oocyte:follicle ratio was found between treatment groups in any of the size ranges (Figure 4.1.12).

4.1.7.3.3 Granulosa cell health

1/ The effect of HR IGF-I and LR3 IGF-I on the health and morphology of bovine early antral follicles in a 4 day *in vitro* culture system.

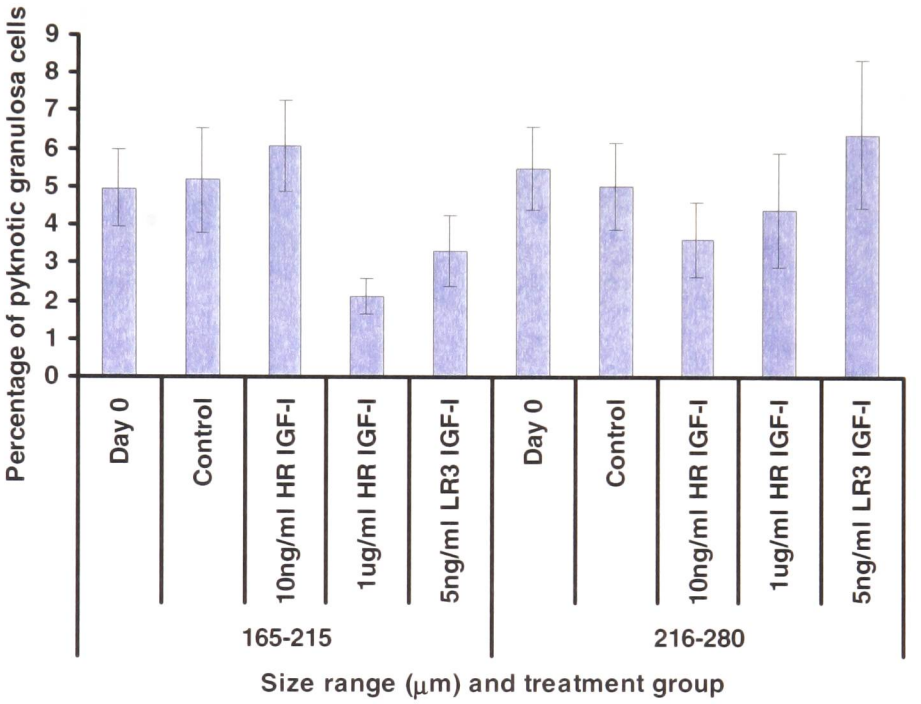


Figure 4.1.13

Effect of HR IGF-I and LR3 IGF-I on granulosa cell health

Granulosa cell health of 2 size ranges of early antral follicles in the following treatment groups: Day 0 (n=20,25), Control (n=30,25), 10ng/ml HR IGF-I (n=31,27), 1μg/ml HR IGF-I (n=26,26) and 5ng/ml LR3 IGF-I (n=29,27). The 'n' numbers are for 165-215μm and 216-280μm size ranges respectively. Values are the mean percentage of pyknotic granulosa cells per treatment group, within each size range, analysed after 4 days of culture.

No significant difference in the percentage of pyknotic granulosa cells was found between treatment groups in any of the size ranges (Figure 4.1.13).

3/ The effect of LR3 IGF-I on the health and morphology of bovine early antral follicles in a 6 day *in vitro* culture system.

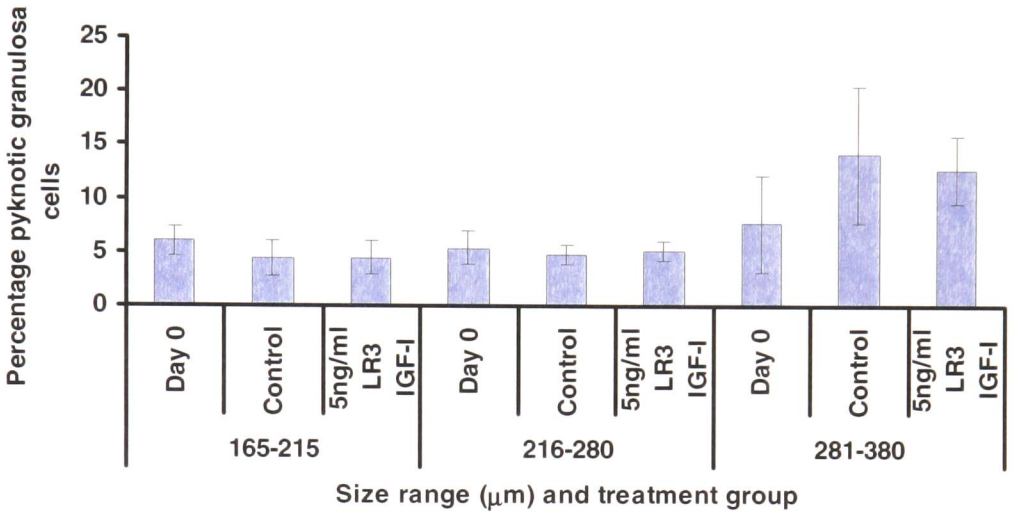


Figure 4.1.14

Effect of LR3 IGF-I on granulosa cell health

Granulosa cell health of 3 size ranges of early antral follicles in the following treatment groups: Day 0 (n=13,13,5) Control (n=18,25,8) and 5ng/ml LR3 IGF-I (25,40,13). The 'n' numbers are for 165-215μm, 216-280μm and 281-380μm size ranges respectively.

Values are the mean percentage of pyknotic granulosa cells per treatment group, within each size range, analysed after 6 days of culture.

No significant difference in the percentage of pyknotic granulosa cells was found between treatment groups in any of the size ranges (Figure 4.1.14).

4.1.8 Discussion

Developing techniques for isolating and culturing the follicles of immature domestic species to a stage where they can produce developmentally competent oocytes and viable offspring presents an enormous challenge. Culture systems have provided a useful tool for elucidating regulatory mechanisms controlling follicle development, but progress has been slow. However, bovine preantral follicles have successfully been maintained in culture (Hulshof *et al.* 1995; McCaffery *et al.* 2000; Saha *et al.* 2000; Thomas *et al.* 2001), with some achieving antrum formation (Gutierrez *et al.* 2000; Itoh *et al.* 2002). Accelerated growth of these immature follicles has been achieved by media supplementation with various hormones and growth factors. There is evidence of a complete intrafollicular IGF system within bovine preantral (Armstrong *et al.* 2002) and antral (Armstrong *et al.* 1998; 2000) follicles, which implies a role for the IGF system throughout follicle development. Previous work has outlined the involvement of IGF-I in follicular development (Giudice 1992). IGF-I has not been found to have a stimulatory role in follicle initiation (Derrar *et al.* 2000; Kezele *et al.* 2002b; Yang and Fortune 2002, see Chapter 3 results). However, recombinant IGF-I has been shown to increase bovine preantral follicle and oocyte diameter *in vitro* (Itoh *et al.* 2002), and LR3 IGF-I has aided the formation of the antral cavity (Gutierrez *et al.* 2000). It is therefore evident that regulation by the IGFBPs of IGF-I is crucial for normal oocyte development in preantral follicles (McCaffery *et al.* 2000).

In the present study, varying concentrations and forms of IGF-I have allowed us to highlight the importance of IGFBPs in IGF regulation at different follicle developmental stages. It has been shown that after six days of culture, the growth of follicles at an immature stage of development (165-215μm) is influenced by direct access to IGF-I (1μg/ml HR IGF-I). However, the more mature follicles (216-380μm) are unaffected by the exposure to IGF-I. This suggests that as follicles grow they are developing and differentiating to a stage where they can withstand increased levels of IGF-I, while immature follicles may undergo inappropriate follicular differentiation caused by untimely exposure to IGF-I. Hence, the presence of IGF-I may not be appropriate until later stages of development. The culture medium used

in ths system was not supplemented with FSH, as IGFs are well known to work in synergy with gonadotrophins to promote follicular development (Spicer *et al.* 1993; Zhao *et al.* 2001) and this study wanted to soley investigate the effects of IGF-I on bovine follicle development. During the early stages of follicle growth stimulation by FSH is not thought to be essential (Dufour *et al.* 1979) however during antral development FSH is vital for the prevention of atresia of antral follicles (Chun *et al.* 1996). Hence, although the follicles in the present study were in the early stages of antral development, therefore not completely dependent on FSH for further growth and development, the addition of FSH to the culture medium may have improved the health and response of the follicles to IGF-I.

Small antral follicles (165-215μm) exhibit an increase in follicle growth and oestradiol production in the presence of 1μg/ml IGF-I. Unlike the immature follicles, the more developed follicles (size ranges 216-280μm and 281-380μm) exhibited no increase in follicle growth, but still registered an increase in oestradiol production in the presence of a high concentration of IGF-I (1μg/ml HR IGF-I). This implies that at this developmental stage the follicles are undergoing rapid differentiation rather then proliferation and respond to the presence of IGF-I in a positive way by increasing oestradiol production and therefore aiding their chances of escaping follicular atresia. The effect of IGF-I has been found, in previous work on bovine ovarian function, to be influenced by the differentiated state of the follicle (Spicer *et al.* 1993). IGF-I at a concentration of 100ng/ml was found to inhibit oestradiol production by granulosa cells from small antral follicles (1-5mm in diameter), whereas oestradiol production was stimulated in granulosa cells from large follicles (≥ 8mm in diameter).

IGF-I interacts primarily with the type I IGF receptor – a homodimer tyrosine kinase receptor – exhibiting a high degree of homology with the insulin receptor. The insulin receptor (IR) and the insulin-like growth factor-I receptor (IGF-IR) belong to the same subfamily of receptor tyrosine kinases. They share a high similarity of structure and intracellular signalling events (Dupont and LeRoith 2001). The biological actions of IGF-I are predominantly mediated by the type I IGF-I receptor.

However, IGF-I is also capable of binding with the closely related insulin receptor (Hwa *et al.* 1999), although this interaction is of a lower affinity than that of insulin for its own receptor. Under certain circumstances, the insulin receptor may mediate some of the biological actions of the IGFs (Hwa *et al.* 1999; LeRoith 2000). The IGF-I receptors would have become overwhelmed with IGF-I when the high dose of IGF-I (1μg/ml HR IGF-I) was present. Therefore, under these conditions the IGF-I may have been able to mediate its actions via the insulin receptors, as well as the type I IGF receptor. This would explain the significant stimulatory effect on the growth of the early antral follicles in the size range 165-215μm and the significant increase in oestradiol production seen in all size ranges of follicles. By contrast, a significant increase in growth was not observed in the follicles grown in media supplemented with LR3 IGF-I. A possible reason for this is that although LR3 IGF-I would have a direct effect (as it does not bind to IGFBPs), the IGF receptors would not become overwhelmed by the LR3 IGF-I due to its low concentration of 5ng/ml. Therefore, these conditions would have only allowed the LR3 IGF-I to facilitate its actions via the type I IGF receptor and not via the insulin receptor as well.

IGF-I is known to stimulate nuclear maturation of cumulus-enclosed oocytes (Lorenzo *et al.* 1994; Sirotkin *et al.* 2000) and improves early embryonic development (Pawshe *et al.* 1998; Sirisathien *et al.* 2003; Sirisathien and Brackett 2003). In contrast to work carried out on bovine preantral follicles (McCaffery *et al.* 2000), IGF-I was not found to have a detrimental effect on oocyte development – in fact it was found to be beneficial in maintaining oocyte health once the follicle had reached a more mature stage of development (281-380μm). During the development of the follicular unit, the oocyte undergoes a progressive series of morphological modifications as it grows and proceeds through the different stages of development. These structural requirements facilitate the increasing energy and nucleic acid synthesis requirements of the developing oocyte and are prerequisite to the oocyte's achievement of meiotic and embryo developmental competence (Fair 2003). The intrinsic quality of the oocyte is the key factor determining the proportion of oocytes that will continue to develop and mature. These experiments demonstrate that when

designing culture systems it is important to consider the developmental stage of the follicular unit as the requirements of the developing follicle are constantly changing.

The role of IGFBP-2 in follicles during early follicle development is unclear. However, it is hypothesised that IGFBP-2 present in the early stages of follicle growth will bind any IGF arriving from the circulation or adjacent follicle, and therefore inhibit the interaction of IGFs and their receptors on the surface of the oocyte and granulosa cells (Armstrong *et al.* 2002). The oocyte and/or its granulosa cells could then access the growth factor when required, by releasing proteases to cleave the IGF from the IGFBP-2. The knowledge that unregulated IGF-I has a detrimental effect on preantral oocyte health (McCaffery *et al.* 2000), and that when you bypass the IGFBP-2 regulatory mechanism in early antral follicles IGF-I can stimulate follicle growth and oestradiol production, highlights that the regulation of IGF-I bioavailability by IGFBP-2 is follicle stage dependent and crucial in the early stages of follicle growth to allow normal follicle and oocyte growth to occur *in vitro*.

In conclusion, this study has investigated the effects of IGF-I on bovine early antral follicle development in a six day, serum-free culture system. It found that the stimulatory effects of IGF-I on follicle proliferation and differentiation were dose and stage dependent. Furthermore, oocyte health was improved by the addition of recombinant IGF-I in the more mature follicles. These results highlight the importance of follicle developmental stage when deciding the best *in vitro* culture conditions. Improved knowledge regarding the regulation of IGFs within follicles will have practical significance in terms of developing *in vitro* growth and maturation systems for oocytes and therefore contribute to the development of techniques for increasing the availability of oocytes from cattle of high genetic merit for subsequent use in embryo transfer procedures.

4.2 Can the insulin receptor be used by IGF-I to mediate its action on early antral bovine follicles?

4.2.1 Introduction

IGF-I and insulin are related structurally and functionally (Rinderknecht and Humbel 1978b; Spicer and Echternkamp 1995). Similarly, the receptor of IGF-I and insulin are also structurally related and both ligands interact with each others receptor's with low affinity (Jones and Clemmons 1995). In section 4.1 it was shown that human recombinant IGF-I at a concentration of 1μg/ml can accelerate the growth rate of early antral follicles in the size range 165-215μm and significantly increase oestradiol production in all of the size ranges (165-380μm). However, this effect was not seen when follicles were cultured with LR3 IGF-I, which does not bind to IGFBPs and can therefore bind directly to the IGF receptors. It was hypothesised that when the high dose of HR IGF-I (1μg/ml) was present the IGF-I receptors would have become overwhelmed with IGF-I. Under these conditions the IGF-I may have been able to mediate its actions via the insulin receptors, as well as the type I IGF receptor. Conversely, although LR3 IGF-I would have a direct effect (would bind directly to the receptors), the IGF receptors would not become overwhelmed by the LR3 IGF-I due to its low concentration of 5ng/ml. Therefore, these conditions would have only allowed for the LR3 IGF-I to facilitate its actions via the type I IGF receptor and not also via the insulin receptor. The following experiment was designed to block the insulin receptor in order to determine the effects of IGF-I that are mediated through its IGF receptors.

4.2.2 Materials and methods

4.2.2.1 Follicle isolation and culture

Early antral follicles (165μm-215μm) were isolated and cultured for 6 days. Half the medium was replaced every second day and the conditioned media was analysed for oestradiol production by hormone assay, as described in Chapter 2.

4.2.2.2 Treatments

Culture medium (control) was prepared as described in 2.3.1. Insulin receptor mouse monoclonal antibody (Vector Laboratories Inc., Peterborough, UK) was used at a 1/10 dilution.

Treatment groups were as follows:

Treatment group	'n' numbers
A Control (no insulin)	12
B 10ng/ml insulin	12
C 10ng/ml insulin + insulin receptor antibody (Insr antibody)	12

Table 4.2.1

Treatment groups and number of replicates

No repeat cultures were carried out as results from initial study showed that the supplementation of the culture medium with insulin receptor mouse monoclonal antibody had no significant effect on growth or oestradiol production suggesting that this is not the most appropriate way to analyse whether IGF-I is able to exert a stimulatory effect on follicle proliferation and differentiation via the IGF receptors alone at this particular stage of follicular development.

4.2.3 Measurement of follicle growth

Every second day follicle diameters were measured using a crossed micrometer (basement membrane to basement membrane) under the dissection microscope.

4.2.4 Measurement of oestradiol production

Detection of oestradiol concentrations in conditioned media was determined by DELFIA, as described in Chapter 2.

4.2.5 Statistical analysis

The data set was normally distributed in accordance with the Anderson-Darling test, variances were homogeneous and treatment groups were randomly assigned, therefore, a powerful parametric test could be used to analysis samples. ANOVA and subsequently the multiple comparison test 'Tukeys' was used after a significant ANOVA to determine where the significant difference existed among the groups. Mean follicle diameters and oestradiol production on day 6 of culture were compared between experimental groups using a repeated measures ANOVA (General Linear Model), with subsequent Tukeys tests to allow for individual comparisons between groups.

4.2.6 Results

4.2.6.1 Follicular growth

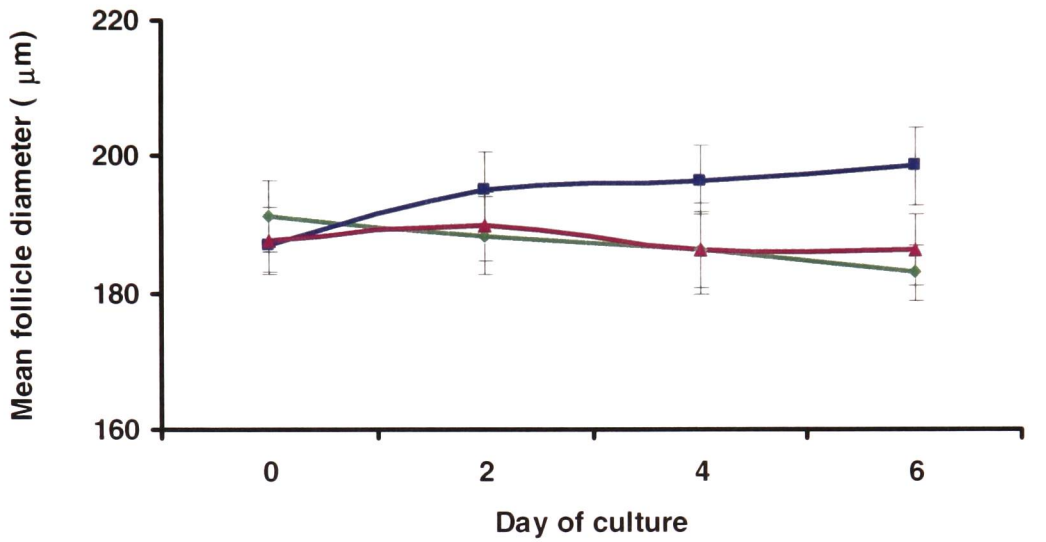


Figure 4.2.1

Follicle growth when follicles were cultured in the presence of insulin, insulin + Insr antibody or no insulin

Growth of early antral follicles in the following treatment groups:

Control (no insulin) = ♦ (n=12), 10ng/ml insulin = ■ (n=12), and 10ng/ml insulin + insulin receptor antibody = ▲ (n=12). Values are mean ± SEM.

No significant difference in follicle growth between days 0 and 6 was found in any of the treatment groups (Figure 4.2.1).

No significant difference in follicle growth was found between any of the treatment groups (Figure 4.2.1).

4.2.6.2 Oestradiol production

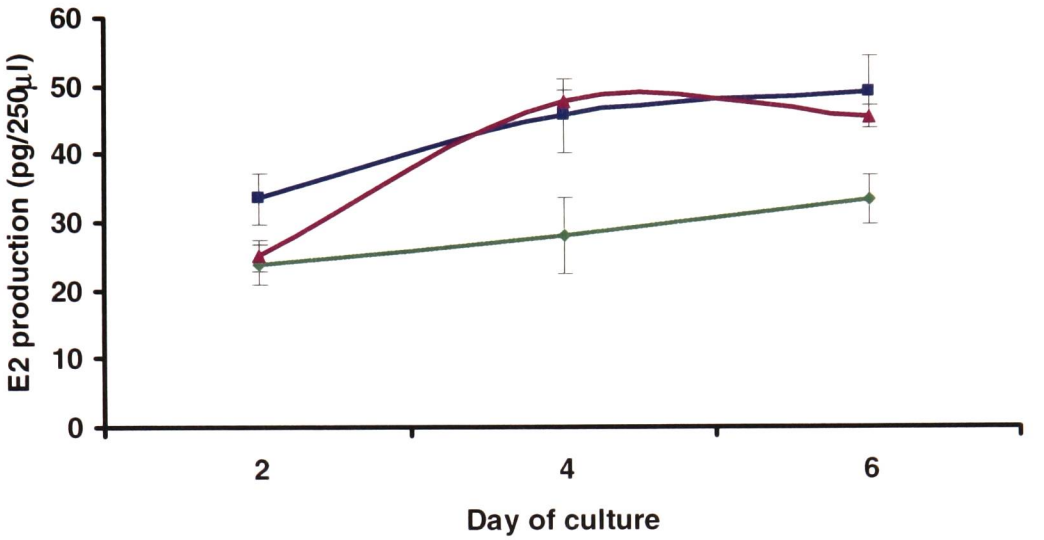


Figure 4.2.2

Oestradiol production when follicles were cultured in the presence of insulin, insulin + Insr antibody or no insulin

Oestradiol production of early antral follicles in the following treatment groups:
Control (no insulin) = ♦ (n=12), 10ng/ml insulin = ■ (n=12), and 10ng/ml insulin + insulin receptor antibody = ▲ (n=12). Values are mean ± SEM.

All groups showed a significant increase in oestradiol production between days 2 and 6 ($p<0.01$) (Figure 4.2.2).

No significant difference in oestradiol production was found between any of the treatment groups (Figure 4.2.2).

4.2.7 Discussion

Both insulin and IGF-I can stimulate each other's receptor with low affinity binding (Jones and Clemmons 1995). In the previous section of this chapter it was hypothesised that when follicles were cultured in media supplemented with a high dose of IGF-I (1μg/ml HR IGF-I), the IGF-I receptors would have become overwhelmed with IGF-I. Therefore, under these conditions the IGF-I might have been able to mediate its actions via the insulin receptors, as well as the type I IGF receptor. Insulin receptor mRNA has been shown to be expressed in the oocyte and granulosa cells of bovine antral follicles (unpublished results from Armstrong, D.G.). This would explain the significant stimulatory effect on the growth of the early antral follicles in the size range 165-215μm, and the significant increase in oestradiol production seen in all size ranges of follicles.

The present study was set up to study the effects of IGF-I acting via only the IGF receptor, through blocking the insulin receptor with the use of an insulin receptor mouse monoclonal antibody. However, from the results it can be seen that the addition of the insulin receptor antibody had no significant effect on growth or oestradiol production, suggesting that the antibody may not have been able to get across the follicular wall. In addition, insulin has been shown in previous studies to exert various effects on ovarian cell types. Proliferation and differentiation of cultured human (Duleba *et al.* 1998) and porcine (Amsterdam *et al.* 1988; Barbieri *et al.* 1983) somatic cells have been stimulated in the presence of insulin. Furthermore, insulin has been shown to have direct effects on bovine ovarian function. It has been shown to stimulate granulosa cell proliferation (Gutierrez *et al.* 1997) and steroidogenesis (Gong *et al.* 1994; Gutierrez *et al.* 1997; Spicer *et al.* 1993), and these effects are influenced by the dose and size of follicle. Hence, by blocking insulin receptors, the beneficial effects of insulin were also being blocked, resulting in poor follicle growth and oestradiol production. Insulin appears to be crucial for normal bovine follicle growth at this stage, so by blocking its actions no conclusion can be reached on the effects of any other factors as the follicles cannot develop in the absence of insulin. This does not appear to be an appropriate way to analyse whether IGF-I is able to exert a stimulatory effect on follicle proliferation and

differentiation via the IGF receptors alone at this particular stage of follicular development. A possible alternative way to address the question of whether IGF-I is mediating its actions via the insulin and IGF-I receptors would be to culture follicles in a high concentration of LR3 IGF-I. This would determine whether at this high dose there is the same stimulatory effect on follicle growth seen in follicles cultured in the presence of a high dose of recombinant IGF-I, bearing in mind that the interaction of LR3 IGF-I with the insulin receptors may be different to that of recombinant IGF-I.

CHAPTER FIVE

The Role of IGF-I on the Regulation of IGFBP-2 Expression

5.1 Introduction

Insulin-like growth factor binding proteins can regulate the availability of IGFs to their target cells by either inhibiting or potentiating their action (Giudice 1992). IGFBPs can sequester extracellular IGFs and hence reduce the number of 'free' IGFs available for specific cell surface receptors; this then inhibits any mitogenic actions of the IGFs. Two mechanisms by which facilitation of the IGF can be increased is by the activity of specific IGFBP proteases and changes in the expression of the IGFBPs (Giudice 1992). At present, mRNA encoding IGFBP-2 to -5 has been detected in bovine follicles (Armstrong and Webb 1997), and IGFBP-2, -4 and -5 mRNA have been found in the ovine follicle (Besnard *et al.* 1996a). IGFBP-2 mRNA has been found to be present in the granulosa cells and oocytes of bovine preantral and early antral follicles (Armstrong *et al.* 2002), as well as the granulosa cells of larger antral follicles (1-8mm) (Armstrong *et al.* 1998). Furthermore, immunoreactive IGFBP-2 has been detected around granulosa cells from bovine follicles at the preantral stage (Armstrong *et al.* 2002). There is a decrease in the concentration of IGFBP-2 in follicular fluid in cows (Armstrong *et al.* 1998) and sheep (Besnard *et al.* 1996a) during late follicular growth, which has been shown to be due to a loss of expression of mRNA encoding IGFBP-2 in granulosa cells in dominant follicles and proteolytic activity (Spicer *et al.* 2001). Likewise, the porcine ovary has been shown to express IGFBP-2, -3, -4 and -5 mRNA (Zhou *et al.* 1996), and – similar to cattle and sheep – IGFBP-2 mRNA and protein levels were also found to decline with an advance in follicle development (Samaras *et al.* 1993).

At various stages of follicle development the regulation of IGF-I bioavailability by IGFBPs is important for normal follicle and oocyte development. The identification of IGF-I binding in bovine preantral follicles (Wandji *et al.* 1992), coupled with the recent detection of expression of type I IGF receptor and IGFBP-2 mRNA from the preantral stage (Armstrong *et al.* 2002), suggests a role for the IGF system in early follicle development. Therefore, it is important to understand the mechanisms involved in regulating the expression of IGFBPs and hence the availability of IGF to its receptors at different stages of growth. Changes in the effects of IGF-I (Monniaux and Pisselet 1992), and the expression of IGFBPs from early to late follicle

development (Monget *et al.* 1996), further supports the view that the need for IGF-I stimulation is dependent on the stage of follicle development.

This current study aimed to identify if IGF-I plays a role in regulating the expression of IGFBP-2 at different stages of follicle growth. In particular, the expression of IGFBP-2 in the oocyte and somatic cells during the crucial transition from late preantral to early antral stages was investigated.

5.2 Materials and methods

5.2.1 Follicle isolation and culture

In Chapter 4 [experiment 2 (The effect of HR IGF-I in the presence or absence of the IGFBP regulatory mechanism)], follicles were cultured to determine the effect of IGF-I on early antral bovine follicular development. Once fixed and mounted on slides they underwent immunocyctochemistry for IGFBP-2. Briefly, early antral follicles in three size ranges (165μm-215μm, 216μm -280μm and 281μm -380μm) were isolated from bovine ovaries and cultured for 2, 4 or 6 days, as described in Chapter 2. Only follicles cultured for 6 days were used for immunocytochemistry for IGFBP-2.

5.2.2 Treatments

Culture medium (control) was prepared as described in 2.3.1. Human recombinant IGF-I (HR IGF-I), which does bind to IGFBPs, was purchased from Sigma Chemicals, Poole, UK. Two doses of HR IGF-I were used: a low dose of 10ng/ml and a high dose of 1μg/ml. The low dose represented a physiological dose that would be able to bind to the IGFBPs and would therefore be regulated by the level of IGFBPs present. The high dose, on the other hand, would swamp the IGFBPs present, allowing IGF-I to bypass any regulatory mechanism of the IGFBPs and hence bind freely to IGF receptors. Under these conditions it is also possible that the IGF-I may be able to bind to the insulin receptors, as well as the IGF receptors, to exert an action.

5.2.3 Detection of IGFBP-2 by immunocytochemistry

Details of the procedure used have been described already by Armstrong et al (1998; 2002). At the end of the culture period, follicles were fixed in 4% paraformaldehyde, embedded in paraffin wax and mounted on slides, as described in Chapter 2. Sections were dewaxed in xylene and rehydrated from ethanol to distilled water, and washed in PBS (2x5 minutes, Sigma Chemicals, Poole, UK). The expression of IGFBP-2 was detected in follicles cultured for 6 days. Antigen retrieval was performed by placing sections (6μm) in 0.01M citrate buffer [2x5 minutes (600W)], and slides

were left for 20 minutes at room temperature, before being washed in phosphate buffer solution (PBS, 2x5 minutes). Endogenous peroxidase was blocked by placing sections in 1% hydrogen peroxide for 10 minutes, followed by washing (2x5 minutes in PBS). The rabbit anti-bovine IGFBP-2 antiserum (Upstate Biotechnology Incorporated, Lake Placid, NY) was diluted 1:200 before use. After probing with primary antibody, the sections were washed and stained using goat anti-rabbit IgG labelled with horseradish peroxidase (1:100) (Sigma Chemicals, Poole, UK). Replacing primary antibody with normal rabbit serum, or saturating the primary antibody with antigen, was performed to detected non-specific binding. The section containing the oocyte nucleolus, or if this was absent, the largest cross-section of the oocyte was used for observations.

5.2.4 Detection of IGFBP-2 by *in situ* hybridisation

At the end of the culture period, follicles were fixed in 4% paraformaldehyde, embedded in paraffin wax and mounted on slides, as described in Chapter 2. Samples were then taken to Roslin Institute were detection of IGFB-2 by *in situ* hybridisation was performed by Jerry Baxter, Karen Troup and Elaine McCullough. Details of the procedure used have been described already by Armstrong *et al.* (1998; 2002). The expression of IGFBP-2 was detected in follicles in all size ranges, cultured for 2, 4 and 6 days. Sections (6μm) were dehydrated, fixed and probed with ^{35}S-labelled IGFBP-2 riboprobe. After the final high stringency wash, the sections were dipped in autoradiographic K2 photographic emulsion (Ilford Limited, Mobberley, Cheshire, UK) and exposed for 6 weeks at 4°C. Sections were developed (Kodak D-19, Edinburgh, UK) and fixed using Hypam fixer (Ilford Limited) before staining in haematoxylin and eosin. The sections were finally mounted in DPX mountant (R. A. Lamb, UK) before microscopic examination using both light- and dark-field illumination.

5.3 Image analysis

Details of the procedure used have been described already by Armstrong et al (1998). The intensity of the *in situ* hybridisation signal was analysed using an NIH-Image analysis system (NIH, Bethesda, MD). The number of graphic pixels occupied by

silver grains (identified by a set grey threshold) within a defined area of the tissue section was counted and presented as a percentage of the total pixel number within the defined area. The section containing the oocyte nucleolus, or if this was absent, the largest cross-section of the oocyte was used for observations. Within each follicle the oocytes and three separate defined areas of granulosa cells were analysed. The hybridisation intensity, therefore, was the percentage of occupied pixels to total pixels within a defined area of the tissue. Background hybridisation intensity, measured with the sense RNA probes, was subtracted from the measurements obtained with the antisense probes to give the final hybridisation signal.

5.4 Statistical assessment

The data set was normally distributed in accordance with the Anderson-Darling test, variances were homogeneous and treatment groups were randomly assigned, therefore, a powerful parametric test could be used to analysis samples.

The intensity of staining for mRNA IGFBP-2 in granulosa cells was compared between days using one-way ANOVA. No significant difference was found between the day of culture within a treatment group; therefore, data for the three different culture days within each treatment group were grouped together. Differences in the intensity of mRNA IGFBP-2 staining in the granulosa cells and oocytes was investigated using one-way ANOVA.

The total number of granulosa cells showing staining and no staining for IGFBP-2 protein was recorded for each follicle. The number of granulosa cells expressing IGFBP-2 protein was expressed as a percentage of the total granulosa cells present for each follicle. Mean percentage of granulosa cells showing expression of IGFBP-2 protein was calculated for each treatment group and then compared between experimental groups using a one-way ANOVA.

The total number of follicles that exhibited staining for IGFBP-2 protein, and those showing no staining for IGFBP-2 protein in their granulosa cells or oocyte in each treatment group, was also recorded. The number of follicles showing staining for

IGFBP-2 protein in their granulosa cells or oocytes was calculated as a percentage of the total number of follicles and displayed as bar charts. Percentages were analysed using a 2-proportion test. The 2-proportions test was chosen as each group was compared to one another and this method tests whether two binomial proportions are significantly different from one another and makes allowances for unbalanced data sets.

5.5 Results

5.5.1 Effect of IGF-I on the level of expression of IGFBP-2 (mRNA) in granulosa cells and oocytes

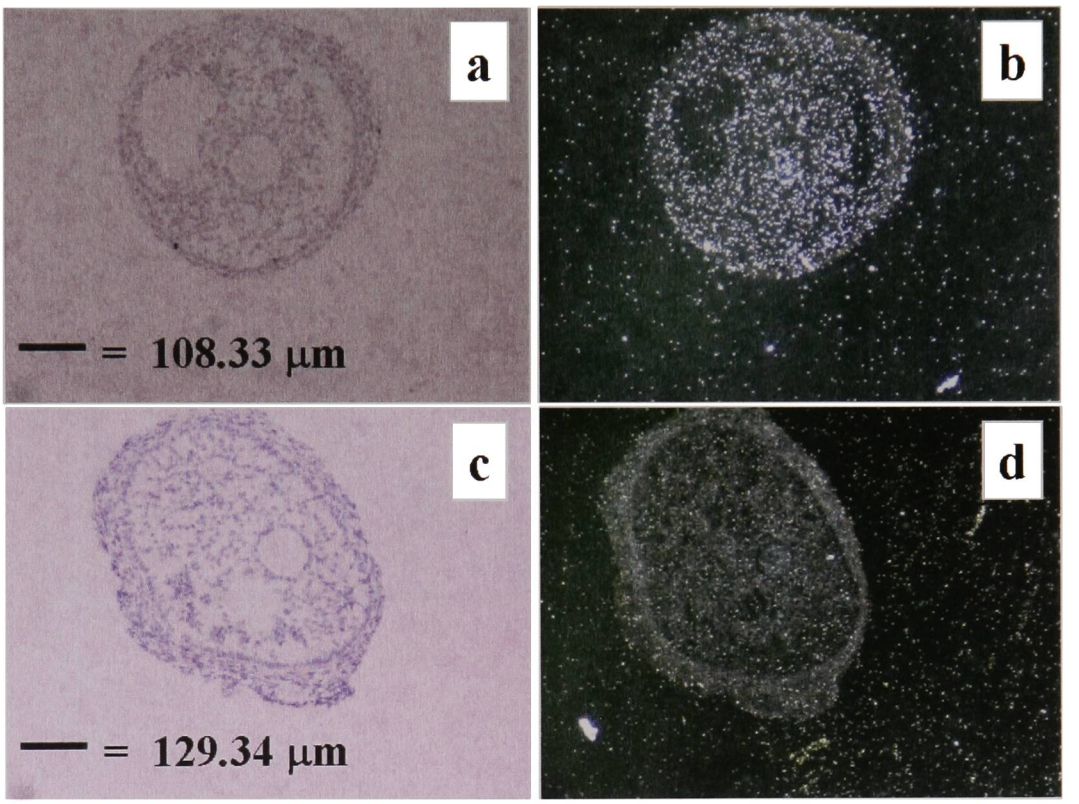


Figure 5.1

mRNA IGFBP-2 expression in bovine follicles

Light-field illumination (**a** and **c**) and dark-field illumination of bovine ovarian follicles on day 6 of culture hybridised with antisense (**a** and **b**) and sense (**c** and **d**) riboprobes for IGFBP-2 detection. Intense staining was found in the granulosa cells and oocytes of follicles hybridized with antisense ^{35}S-IGFBP-2 riboprobe (**b**). No staining was found in follicles hybridized with sense ^{35}S-IGFBP-2 riboprobe (**d**).

mRNA IGFBP-2 was observed in the granulosa cells and oocytes of follicles on all days, in all treatment groups and in all size ranges (Figure 5.1).

Effect of day on mRNA IGFBP-2 expression

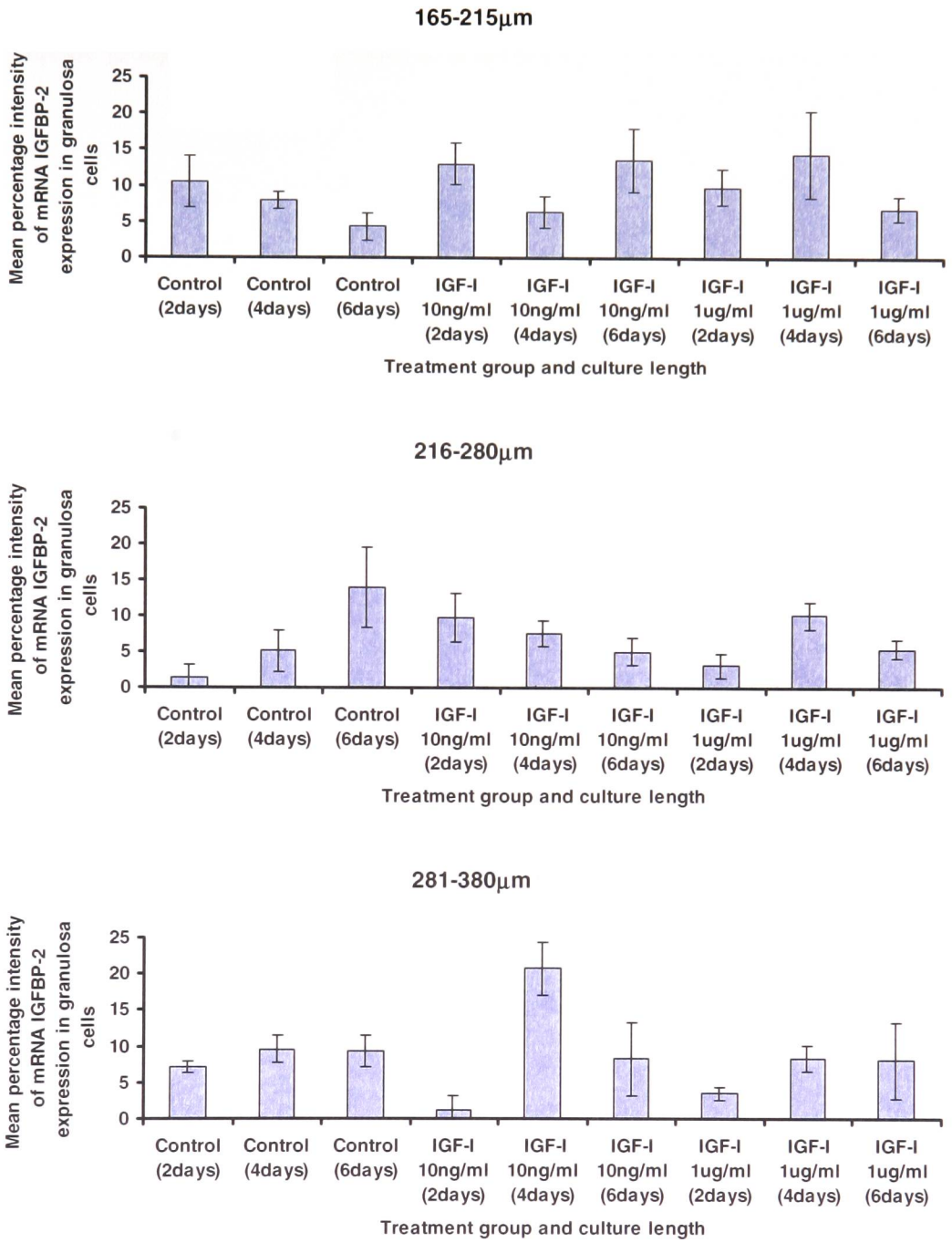


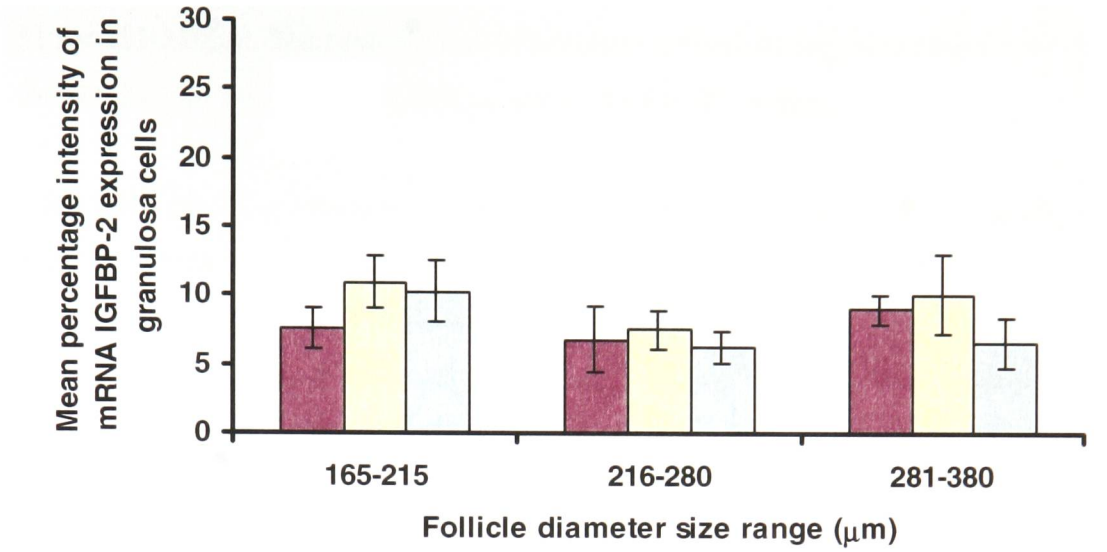
Figure 5.2

The intensity of the *in situ* hybridisation signal for mRNA IGFBP-2 in the granulosa cells of 3 different size ranges and on 3 different days of culture

The total number of separate defined areas of granulosa cells analysed in each size range was n= 54 for 165-215μm, n= 54 for 216-280μm and n= 51 for 281-380μm.

No significant difference was found between the day of culture within a treatment group (Figure 5.2); therefore, data from the three different culture days within each treatment group were grouped together (Figure 5.3).

Effect of treatment on mRNA IGFBP-2 expression



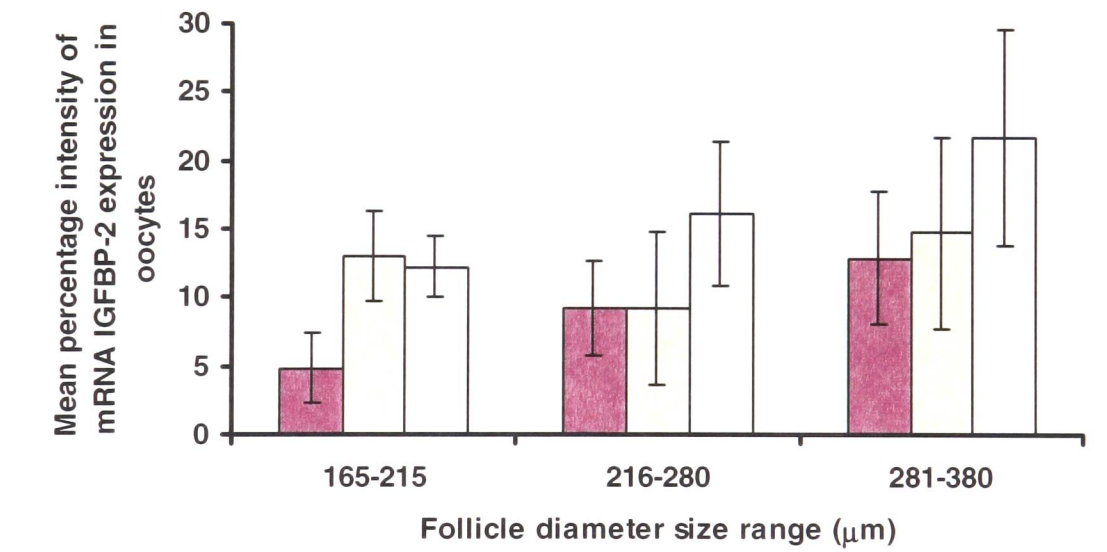


Figure 5.3

The intensity of the *in situ* hybridisation signal for mRNA IGFBP-2 in the oocytes and granulosa cells of 3 different size ranges

Control = , IGF-I 10ng/ml = and IGF-I 1μg/ml = .

Values are the mean percentage intensity of mRNA IGFBP-2 expression in granulosa cells per follicle per treatment group, and the mean percentage intensity of mRNA IGFBP-2 expression in oocytes per treatment group, within each size range.

The total number of separate defined areas of granulosa cells analysed in each size range for figures 5.2 and 5.3 is n= 54 for 165-215μm, n= 54 for 216-280μm and n= 51 for 281-380μm. The total number of oocytes analysed in each size range is n= 18 for 165-215μm, n= 18 for 216-280μm and n= 17 for 281-380μm.

mRNA IGFBP-2 was observed in the granulosa cells and oocyte of follicles on all days, in all treatment groups and in all size ranges (Figure 5.1). Initial analysis showed that there was no significant difference in hybridisation intensity between days (Figure 5.2) or treatment groups (Figure 5.3). Therefore, the effect of IGF-I on IGFBP-2 protein production was investigated.

5.5.2 Effect of IGF-I on the percentage of follicles expressing IGFBP-2 (protein) in their granulosa cells

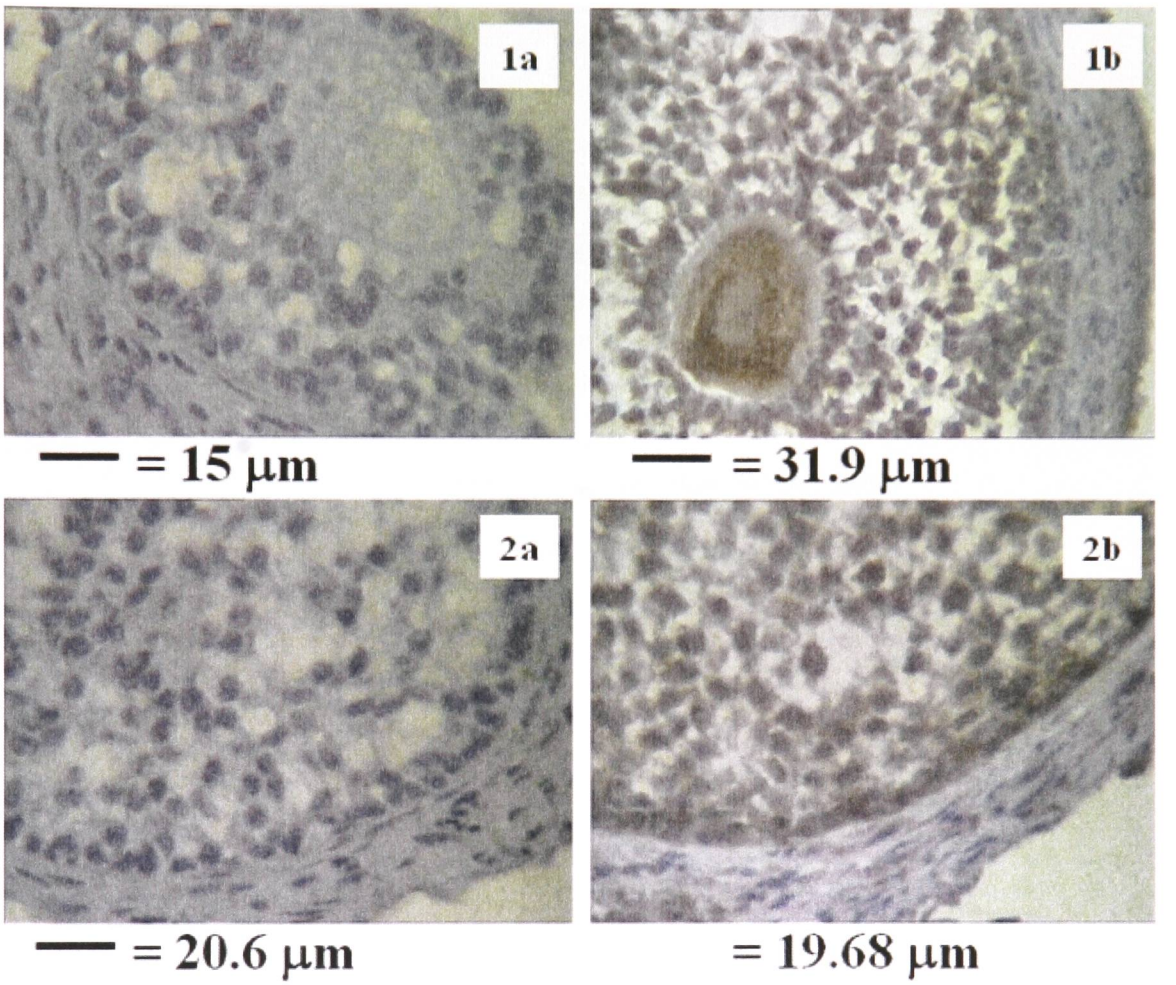


Figure 5.4

IGFBP-2 immunoreactivity in bovine follicles

Histological sections of bovine ovarian follicles on day 6 of culture after immunocytochemistry with (**a**) normal rabbit serum or (**b**) an antibody raised against bovine recombinant insulin-like growth factor binding protein-2 (IGFBP-2). **1a** and **1b** = oocyte, granulosa and theca cells; **2a** and **2b** = granulosa and theca cells.

Protein IGFBP-2 was observed in the granulosa cells and oocyte of follicles in all treatment groups and in all size ranges (Figure 5.4).

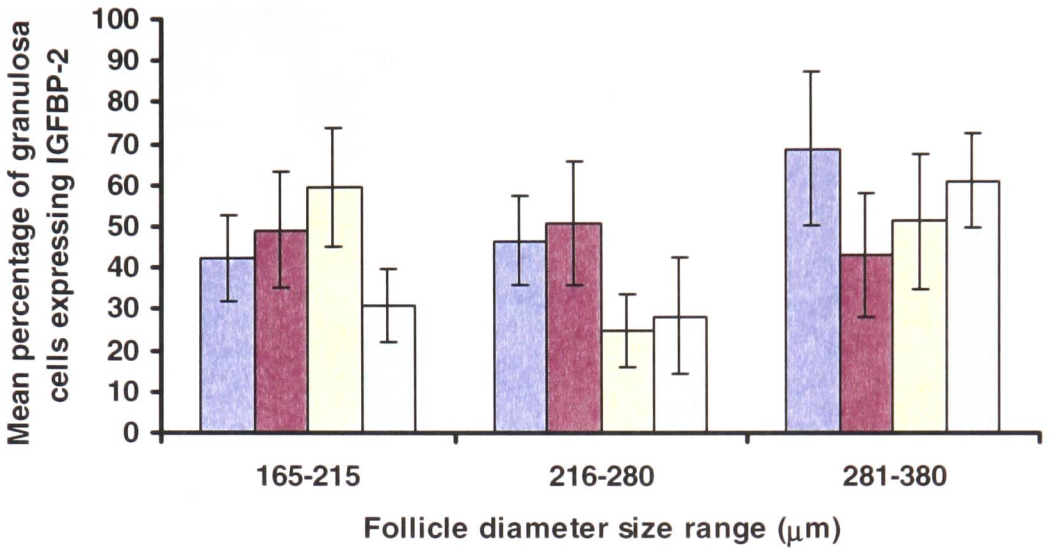


Figure 5.5

Effect of IGF-I on the percentage of granulosa cells expressing IGFBP-2 (protein) on day 6 of culture

Day 0 = , Control = , IGF-I 10ng/ml = and IGF-I 1μg/ml = .

Values are the mean percentage of granulosa cells showing staining for IGFBP-2 for each treatment group within each size range.

The total number of follicles in each size range for figures 5.5, 5.6 and 5.7 was n= 39 for 165-215μm, n= 38 for 216-280μm and n= 31 for 281-380μm.

No significant difference was found between treatment groups in the mean percentage of granulosa cells showing staining for IGFBP-2 protein (Figure 5.5).

No significant different was found between treatment groups in the mean percentage of granulosa cells exhibiting staining for IGFBP-2 protein (Figure 5.5). Therefore, the actual total number of follicles showing expression for IGFBP-2 was calculated as a percentage of the total number of follicles analysed, and comparisons were made between treatment groups.

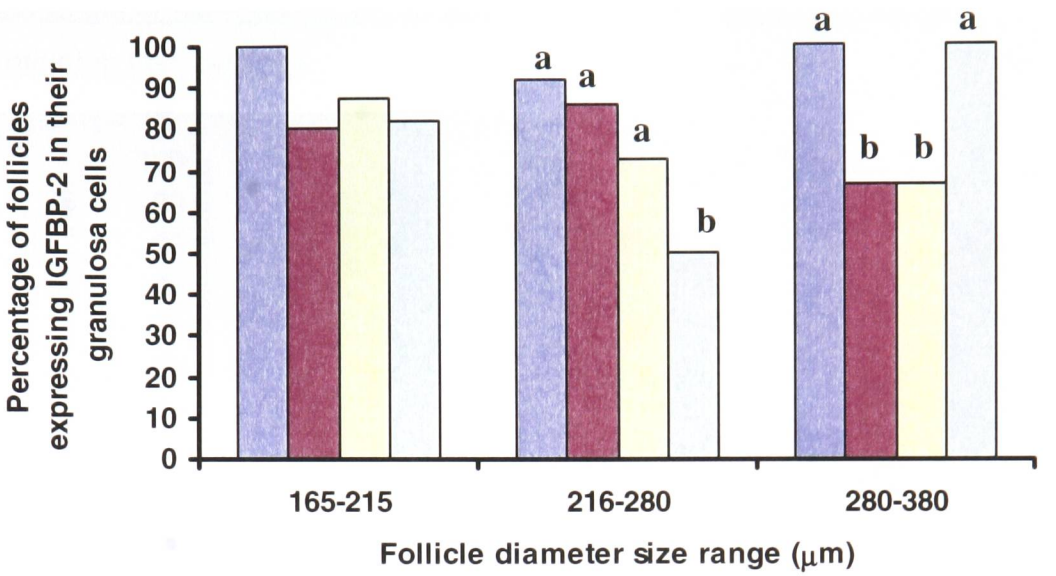


Figure 5.6

Effect of IGF-I on the percentage of follicles expressing IGFBP-2 (protein) in their granulosa cells on day 6 of culture

Day 0 = , Control = , IGF-I 10ng/ml = , IGF-I 1μg/ml = .

The total number of follicles in each size range is n= 39 for 165-215μm, n= 38 for 216-280μm and n= 31 for 281-380μm.

Values are the mean percentage of follicles showing staining for IGFBP-2 in their granulosa cells for each treatment group within each size range.

No significant differences in the percentage of follicles expressing IGFBP-2 in their granulosa cells were found in the smallest size range of follicles (165-215μm). However, a significant decrease in IGFBP-2 expression was found in medium follicles cultured in 1μg/ml IGF-I compared to the level of expression seen in the Day 0 follicles ($p<0.05$). Furthermore, in the largest size range (281-380μm), there was a significant decrease in the number of follicles expressing IGFBP-2 in the control and 10ng/ml IGF-I treatment groups compared to that seen in the Day 0 experimental group ($p<0.05$). Follicles cultured with 1ug/ml IGF-I were found to maintain the level of expression seen in the Day 0 follicles, and therefore had a significantly higher percentage of follicles expressing IGFBP-2 than those seen in the control and 10ng/ml IGF-I treatment groups ($p<0.05$) (Figure 5.6).

5.5.3 Effect of IGF-I on the percentage of follicles expressing IGFBP-2 (protein) in their oocytes

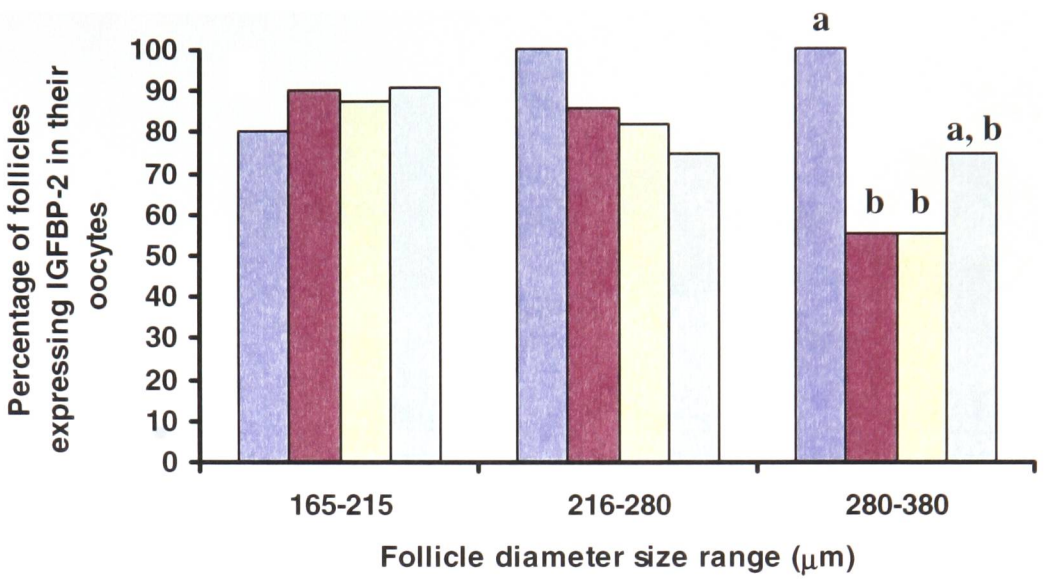


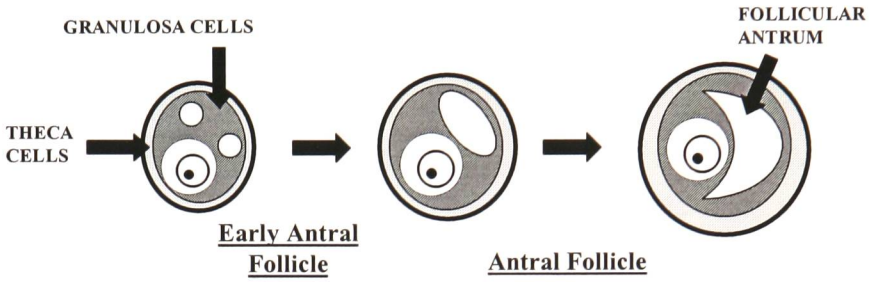
Figure 5.7

Effect of IGF-I on the percentage of follicles expressing IGFBP-2 (protein) in their oocytes on day 6 of culture

Day 0 = , Control = , IGF-I 10ng/ml = , IGF-I 1μg/ml = .

'n' numbers are the same as in Figure 5.3. Values are the mean percentage of follicles showing staining for IGFBP-2 in their oocytes for each treatment group within each size range.

No significant difference was found between treatment groups in the percentage of follicles expressing IGFBP-2 in their oocytes in follicle groups with a small and medium size range (165-215μm and 216-280μm). However, a significant decrease in IGFBP-2 expression was found in the largest size range (281-380μm) of follicles cultured in the control or 10ng/ml IGF-I treatment groups ($p<0.01$) (Figure 5.7).



	191μm ± 2.64	246μm ± 3.26	313μm ± 5.2
% of granulosa cells expressing IGFBP-2	No effect	No effect	No effect
% of follicles expressing IGFBP-2 in their granulosa cells	No effect	↓ when cultured with 1 μg/ml HR IGF-I	↓ when cultured with control or 10 ng/ml HR IGF-I
% of follicles expressing IGFBP-2 in their oocytes	No effect	No effect	↓ when cultured with control or 10ng/ml HR IGF-I

Figure 5.8

Summary of the effects of human recombinant IGF-I on IGFBP-2 (protein) expression in early antral bovine ovarian follicles cultured *in vitro*

5.6 Discussion

Previous work has outlined the involvement of IGFBP-2 in the regulation of IGF-I bioavailability (Armstrong *et al.* 1998; Armstrong *et al.* 2002; Monget *et al.* 2002; Perks and Wathes 1996; Yuan *et al.* 1998). It has been hypothesised that IGFBP-2 present in the early stages of follicle growth will bind any IGF arriving from the circulation or adjacent antral follicles and regulate its access to the type I IGF receptors in the oocyte and granulosa cells (Armstrong *et al.* 2002). This study has confirmed that IGFBP-2 (mRNA and protein) is expressed in the oocyte and granulosa cells of early antral follicles. Furthermore, IGF-I has been identified as a growth factor that plays a role in controlling the expression of IGFBP-2, and this regulatory effect appears to be dependent on the stage of follicle development.

Armstrong *et al.* (1998) showed that *in vitro*, FSH strongly inhibits expression of IGFBP-2 mRNA by bovine granulosa cells taken from antral follicles (4-8mm in diameter). Similarly, FSH was shown to strongly decrease the expression of IGFBP-4 and -5 by rat granulosa cells (Liu *et al.* 1993). By contrast, FSH was found to have no effect on IGFBP-2 production in ovine granulosa cells (Armstrong *et al.* 1996). Follicles can grow to the early antral stage in the absence of gonadotrophins (Awotwi *et al.* 1984; Gong *et al.* 1996), and are not dependent upon gonadotrophin control until later in antral development. Although there is some evidence to suggest that FSH might have a role in the rate of preantral development in sheep (Campbell *et al.* 2004), it seems unlikely that FSH would be involved in playing a crucial role during these early stages of development.

The correct regulation of IGFBPs during follicle development is essential to ensure that the follicle is not under or over exposed to circulating IGFs. IGFBP expression in larger antral follicles than used in the current study have been found to be influenced by the presence of various hormones and growth factors (Chamberlain and Spicer 2001; Sakal *et al.* 1992; Voge *et al.* 2004). FSH in the presence or absence of insulin was found to increase IGFBP-2 mRNA but not change IGFBP-2, -4 or -5 mRNA levels in granulosa cells of antral follicle 1-5 mm in diameter. Furthermore, oestradiol was found to increase IGFBP-2 mRNA in the presence of

insulin, and LH increased IGFBP-3 mRNA levels. In larger follicles (7-9 mm) insulin was found to increase IGFBP-2 gene expression, while oestradiol decreased IGFBP-5 mRNA levels (Voge *et al.* 2004). In a similar study, the production of IGFBP-2 and -5 by granulosa cells was inhibited by insulin, with EGF and bFGF further enhancing insulin's inhibitory actions (Chamberlain and Spicer 2001). These results indicate that the expression of IGFBPs is differentially regulated by various stimuli, and hence allows different levels of regulation for the bioavailability of IGFs. IGF itself has been shown to play a role in the control of ovine granulosa cell IGFBP-2 production, where both FSH and IGF-I were necessary for maximum production of IGFBP-2 (Armstrong *et al.* 1996). Grimes and Hammond (1992) showed that IGF-I in a dose-dependent manner significantly stimulated the production of IGFBP-2 and -3 in granulosa cells taken from 4-6 mm diameter porcine follicles. Furthermore, the synthetic analogue, LR3-IGF-I, which has a low affinity for IGFBPs, was shown to have a significantly greater potency in stimulating the production of these IGFBPs than IGF-I. These results suggest that IGFBP production is regulated via the type I IGF-I receptor, and that an increase in IGF activity stimulates the production of IGFBPs, which in turn reduces the bioavailability of the IGFs. The results found in this current study (summarised in Figure 5.8) support the findings that IGF-I can stimulate IGFBP-2 expression in a dose-dependent manner. It was found that the percentage of follicles expressing IGFBP-2 in their granulosa cells in the largest size group decreased when IGF-I was absent or the concentration present was low. By contrast, when the follicles were cultured in a high concentration of IGF-I the high level of expression of IGFBP-2 found in the Day 0 follicles was maintained. Oestradiol in the presence of insulin has been shown previously to increase IGFBP-2 mRNA expression in granulosa cells of antral follicles (1-5mm in diameter) (Voge *et al.* 2004), and interestingly in the present study there was found to be a significant increase in oestradiol production in the follicles cultured in the high dose of IGF-I (see Chapter 4 results), which also exhibited a high level of IGFBP-2 expression. However, it is unclear whether the increase in oestradiol production in the present study was directly responsible for the increase in IGFBP-2 expression. The results presented here suggest that at this stage in development the granulosa cells have differentiated enough to be able to respond

to the action of IGF-I by adjusting the expression of IGFBP-2, possibly by a negative feedback mechanism. Furthermore, the oocyte appears to have developed to a stage where it can also modulate the expression of IGFBP-2 to regulate IGF bioavailability.

For the ovarian follicle to develop properly a complex set of reciprocal interactions between the oocyte and granulosa cells is required. Recent evidence has shown that the influence of the oocyte on granulosa cell changes and vice versa; it alters with the progression of their development (Latham *et al.* 2004). *In vivo* (Armstrong *et al.* 2001) and *in vitro* (McCaffery *et al.* 2000) studies have shown that inappropriate exposure of the oocyte to IGF-I within preantral follicles is detrimental to oocyte development during early follicle growth. However, in the present study, the expression of IGFBP-2 in follicles in the size range 165-215μm was found not to respond to the presence of IGF. This implies that the follicles are still at an immature stage of development, where the oocyte has not yet advanced and the granulosa cells have not differentiated to a stage of growth where a mechanism of regulation for IGFBP-2 has developed. However, when a high concentration of IGF-I was present, follicles in the size range 216-280μm were able to respond to IGF-I by modulating the expression of IGFBP-2. It could be hypothesised that at this high concentration, IGF-I would not be regulated as it would be able to bypass the IGFBPs and thus have a direct action on the follicle. This inappropriate exposure of the follicle to IGF-I may allow the granulosa cells to accelerate in their development to a stage where they become differentiated enough to respond to IGF-I by modulating the expression of IGFBP-2. Conversely, the oocyte remains unaffected by this high concentration, suggesting that at this stage of growth the actions of IGF-I on granulosa cells and the oocyte are independent of each other, and are instead influenced by the developmental stage and size of the follicle.

In conclusion, this work has shown that IGF-I can play a role in regulating its own bioavailability by influencing the expression of IGFBP-2. It is now clear that the biological actions of IGF depend in part on the ability of specific proteases to break down the IGF/IGFBP complex and/or the level of expression of the IGFBPs (Giudice

1992). The results from this study suggest that IGF-I may regulate the expression of IGFBP-2 through a negative feedback mechanism, which again is stage dependent. The control of local growth factors by regulatory mechanisms needs to be more fully investigated, as it appears to be key to developing an *in vitro* culture system that will accelerate follicle development without causing precocious differentiation to occur. The culture medium used in this system was not supplemented with FSH, as IGFs are well known to work in synergy with gonadotrophins to promote follicular development (Spicer *et al.* 1993; Zhao *et al.* 2001) and this study wanted to solely investigate the effects of IGF-I on bovine follicle development.

CHAPTER SIX

Identification of IGFBP-2 Proteolytic Degradation During Follicular Development in Cattle

6.1 Introduction

The bioavailability of IGFs is regulated by a family of IGF binding proteins (IGFBPs) that bind IGF with higher affinity than the IGF-I and -II receptors. Hence, effects of IGF can be regulated at the level of the target cells (Jones and Clemmons 1995). The IGFBP family consists of at least six structurally related proteins (IGFBP-1 to -6) with high but different affinities for IGF-I and IGF-II (Jones and Clemmons 1995), and a number of IGFBP-related proteins with lower affinities for IGFs (Kim *et al.* 1997; Nicholas *et al.* 2002). The affinity of IGFBPs for IGFs is regulated by a number of post-translational modifications, including phosphorylation, glycosylation and proteolytic cleavage (Firth 1998).

Follicular growth is associated with changes in IGFBP concentrations more than with changes in the IGF-I and -II ligands (Spicer and Echternkamp 1995; Stewart *et al.* 1996). At specific developmental stages during the growth of the follicle the relative ratio of the IGFs to IGFBPs varies, allowing cell-specific growth promoting effects of the IGFs. Proteases are enzymes that catalyse the breakdown of peptide bonds by the process of proteolysis, and the proteolytic cleavage (degradation of the IGF/IGFBP complex) of IGFBPs by proteases generally results in reduced affinity of the fragments for IGFs (Cwyfan-Hughes *et al.* 1997; Rajaram *et al.* 1997). Members of several classes of proteases have shown the ability to degrade IGFBPs, including kallikreins, cathepsins, PAPP-A and matrix metalloproteinases (Firth and Baxter 2002; Rajah *et al.* 1995). Proteolysis of IGFBPs has been detected in follicular fluid from humans (Chandrasekher *et al.* 1995; Cwyfan-Hughes *et al.* 1997), cattle (Rivera *et al.* 2001; Spicer *et al.* 2001), ewes (Besnard *et al.* 1996b; 1997; Mazerbourg *et al.* 1999), mares (Bridges *et al.* 2002) and pigs (Besnard *et al.* 1997), and the amounts and activity of these enzymes change during follicle development. Expression of the mRNAs encoding IGFBP-2 to -5 have been found in bovine follicles and expression of IGFBP-2, -4 and -5 in ovine follicles (Webb *et al.* 1999). In the cow (Roberts and Echternkamp 2003) and in the ewe (Besnard *et al.* 1996a; Monget *et al.* 1993) follicular growth is characterised by an increase in IGFBP-3 and a decrease in IGFBP-2, -4 and –5 levels. By contrast, follicular atresia is associated with a slight decrease in IGFBP-3 levels in the ewe (Monget *et al.* 1993) and a large increase in

IGFBP-2, -4 and –5 in both the ewe and heifer (Monget *et al.* 1993; Roberts and Echternkamp 2003).

The decrease in IGFBP-2 concentration in follicular fluid in cows (Armstrong *et al.* 1998) and sheep (Besnard *et al.* 1996a) during follicular growth has been shown to be due to a loss of expression of mRNA encoding IGFBP-2 in granulosa cells in dominant follicles (Armstrong *et al.* 1998). However, recent data has confirmed that the decrease in IGFBP-2 is also partly due to an increase in IGFBP-2 proteolytic degradation (Besnard *et al.* 1996b; Monget *et al.* 2003; Spicer *et al.* 2001). This in turn would increase the available 'free' IGF and hence increase FSH responsiveness of the granulosa cells. In contrast to the expression of the mRNA encoding IGFBP-2 in granulosa cells, the expression of mRNA encoding IGFBP-4 in thecal tissue does not change during follicular growth (Armstrong *et al.* 1998; Besnard *et al.* 1996a). The decrease in IGFBP-4 concentration in follicular fluid during the development of the follicle is due entirely to a corresponding increase in the activity of a specific IGFBP-4 protease (Besnard *et al.* 1996a). Indeed, the finding that IGFBP-4 proteolytic activity is higher in dominant, oestrogen-active follicles than in subordinate follicles of the same cohort suggests that the IGFBP-4 protease plays a role in the establishment of ovarian follicular dominance in cattle (Rivera *et al.* 2001).

During follicle development, IGFBP-2 and type I IGF receptor have been detected in granulosa cells and oocytes of bovine preantral (Armstrong *et al.* 2000; 2002) and antral follicles (Armstrong *et al.* 1998; 2000, see Chapter 5 results). Furthermore, the oocyte, cumulus and mural granulosa cells of preovulatory follicles have also been shown to express IGFBP-2 and type I IGF receptor (Nuttinck *et al.* 2004), implying that a fully functional IGF system is present throughout the majority of the bovine follicular growth phase. Evidence of a small, yet significant, amount of IGFBP-2 proteolytic activity in bovine subordinate (>1 mm in diameter) and preovulatory follicles was confirmed by the appearance of a 12-kDa breakdown product of ^{125}I-IGFBP-2 (Spicer *et al.* 2001). It was found that large dominant follicles had a significantly lower IGFBP-2 proteolytic activity than the subordinate small and large

follicles (Spicer *et al.* 2001). Furthermore, proteolysis of ^{125}I-labelled IGFBP-2 was negatively correlated with follicular fluid oestradiol and positively correlated with follicular fluid levels of IGFBP-2 (Spicer 2004). IGFBP-2 proteolytic activity during follicle growth has also been detected in horses (Bridges *et al.* 2002), sheep (Besnard *et al.* 1996b) and pigs (Besnard *et al.* 1997). However, in contrast to cattle, there is a small increase in IGFBP-2 proteolytic activity during follicle growth in sheep (Besnard *et al.* 1996b) and pigs (Besnard *et al.* 1997), but no change during follicle growth in horses (Bridges *et al.* 2002). Recently, Pregnancy-Associated Plasma Protein-A (PAPP-A), which is known to be a IGFBP-4 protease in a variety of species (human: Conover *et al.* 2001; ovine, bovine, porcine and equine: Mazerbourg *et al.* 2001), has now been identified as a protease present in bovine, equine and porcine follicular fluid capable of cleaving IGFBP-2 (Gerard *et al.* 2004; Monget *et al.* 2003).

FSH in human (Adachi *et al.* 1995) and rat (Liu *et al.* 1993; Fielder *et al.* 1993) granulosa cell cultures has been shown to induce proteolysis, while IGFs have been found to block FSH-induced proteolysis of IGFBP-5 in cultured rat granulosa cells (Fielder *et al.* 1993) and to block IGFBP-3 proteolytic degradation in porcine granulosa cell cultures (Grimes and Hammond 1994). The mechanism by which IGF inhibits protease activity in these culture systems is unclear. However, mediation by IGF receptors is unlikely as antagonism of receptor action was found not to alter intrinsic degradation of IGFBP-3 in porcine granulosa cells. Furthermore, IGF-I inhibited degradation in cell-free conditioned medium, which suggests direct stabilisation of IGFBP-3 via binding of IGFs, whilst LR3 IGF-I attenuated IGFBP-3 degradation even though it has a low affinity for IGFBPs. IGFs may, therefore, inhibit proteases directly (Grimes and Hammond 1994). Contrary to these results, data from human granulosa cell cultures shows that binding of IGF to IGFBP-4 actually increases the susceptibility of IGFBP-4 to proteolysis (Iwashita *et al.* 1998) and similarly, incubation of IGF-I and follicular fluid from bovine, porcine and equine preovulatory follicles enhances IGFBP-2 (Gerard *et al.* 2004; Monget *et al.* 2003) and IGFBP-4 (Mazerbourg *et al.* 2000) degradation.

Oocytes have been shown recently to play a role in regulating and controlling their own development. Indeed, oocytes may secrete many proteins during their development whose functions, at the present time, remain unknown (Eppig *et al.* 2002). The identification and characterisation of oocyte-secreted factors is crucial to resolving the mechanisms by which the oocyte can orchestrate follicular development. The presence of mRNA encoding IGF receptors and IGFBP-2 in the granulosa cells and oocyte of bovine follicles from the preantral stage and onwards highlights the importance of the IGF system throughout follicular development (Armstrong *et al.* 1998; 2000; 2002; Nuttinck *et al.* 2004, see Chapter 5 results). The production of specific IGFBP proteases by the oocyte and/or granulosa cells would provide a regulatory mechanism whereby the oocyte and/or granulosa cells can regulate their own exposure to IGFs. Furthermore, recent studies in cattle have demonstrated that the oocyte can secrete paracrine growth factors that can interact with the surrounding cumulus cells to modulate their gonadotrophin and IGF-stimulated steroidogenesis (Glister *et al.* 2003; Li *et al.* 2000).

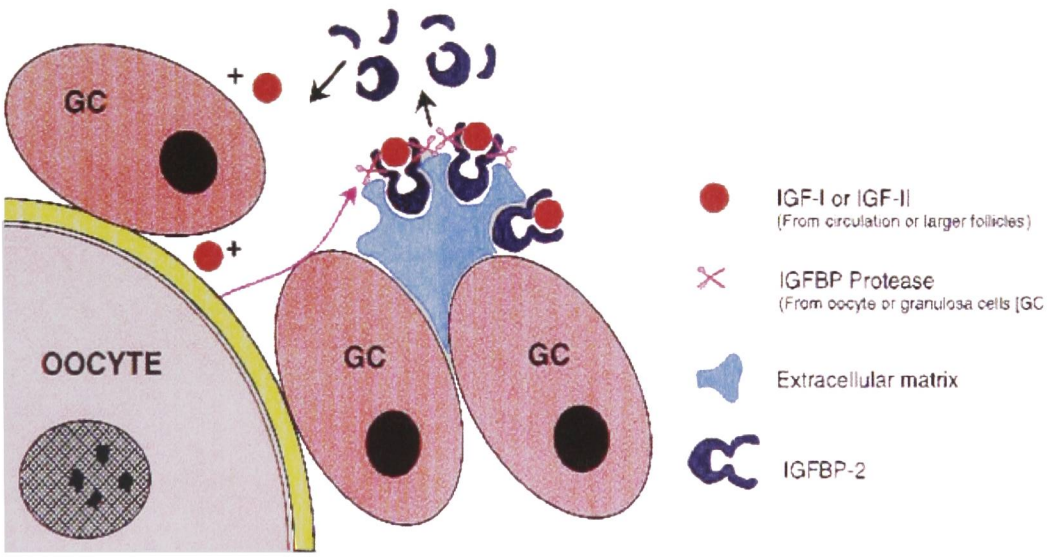


Figure 6.1

Diagrammatic representation of hypothetical mechanism by which bovine oocytes and granulosa cells can regulate their own exposure to IGFs by the production of specific IGFBP proteases

Previous studies have shown that preovulatory follicular fluid contains proteases capable of degrading IGFBP-2 (Spicer *et al.* 2001; Bridges *et al.* 2002; Besnard *et al.* 1996b; Besnard *et al.* 1997); however the cell type producing these proteases is unknown. With this knowledge, the present study hypothesised that the bovine oocyte, cumulus and/or mural granulosa cells were capable of secreting IGFBP-2 proteases at specific stages of development to increase IGF bioavailability, and hence IGF actions. This regulatory mechanism would allow the oocyte and granulosa cells to regulate their own exposure to IGFs (Figure 6.1). Therefore, the aim of this work was to identify the occurrence of IGFBP-2 proteolysis as a result of the incubation of intact IGFBP-2 with different bovine follicular compartments. Furthermore, the study also looked at whether IGF-I or FSH have an effect in modulating IGFBP-2 proteolytic degradation.

6.2 Materials and methods

6.2.1 Collection of follicular fluid, oocyte-cumulus complexes, denuded oocytes and mural granulosa cells

Bovine follicular fluid, oocyte-cumulus complexes, denuded oocytes and granulosa cells were obtained, as described in Chapter 2.

6.2.2 Production of concentrated conditioned medium

To rule out the possibility that a dilution effect was occurring because bovine oocyte-cumulus complexes and denuded oocytes were only producing proteases capable of cleaving IGFBP-2 in very low concentrations, x10 concentrated samples of oocyte-cumulus complex, denuded oocyte and mural granulosa cell conditioned media were prepared before being incubated with intact IGFBP-2. Oocyte-cumulus complex (2.5 oocytes/μl), denuded oocyte (2.5 oocytes/μl) and granulosa cell concentrated conditioned media were obtained by collection of 100ul of conditioned media for each treatment group, which was lyophilised and then reconstituted in 10μl dH_2O to give a x10 concentrate.

6.2.3 Measurement of IGFBP-2 cleavage

IGFBP-2 (31 kDa) (1ng/ml) (GroPep Limited, Adelaide, Australia) was incubated for 20 hours with oocyte-cumulus complex (2.5 oocytes/μl), denuded oocyte (2.5 oocytes/μl), granulosa cells (6,900/μl) (measured using Improved Neubauer haemocytometer, Hawksley, England) or concentrated conditioned media. Intact IGFBP-2 alone and follicular fluid (previously shown to cleave IGFBP-2 (Spicer *et al.* 2001)) cultured with intact IGFBP-2, were used as negative and positive controls respectively. To investigate if FSH and/or IGF-I had the ability to enhance or inhibit the degradation of IGFBP-2, FSH (300mIU/ml) (Puregon, Organon) or human recombinant IGF-I (10ng/ml) (Sigma Chemicals, Poole, UK) were incubated with the IGFBP-2 and conditioned media. Proteolytic activity was analysed by immunoblotting and western ligand blotting as described in Chapter 2.

6.3 Results

6.3.1 Detection of proteolysis of IGFBP-2 by oocyte-cumulus complexes, denuded oocytes and mural granulosa cells

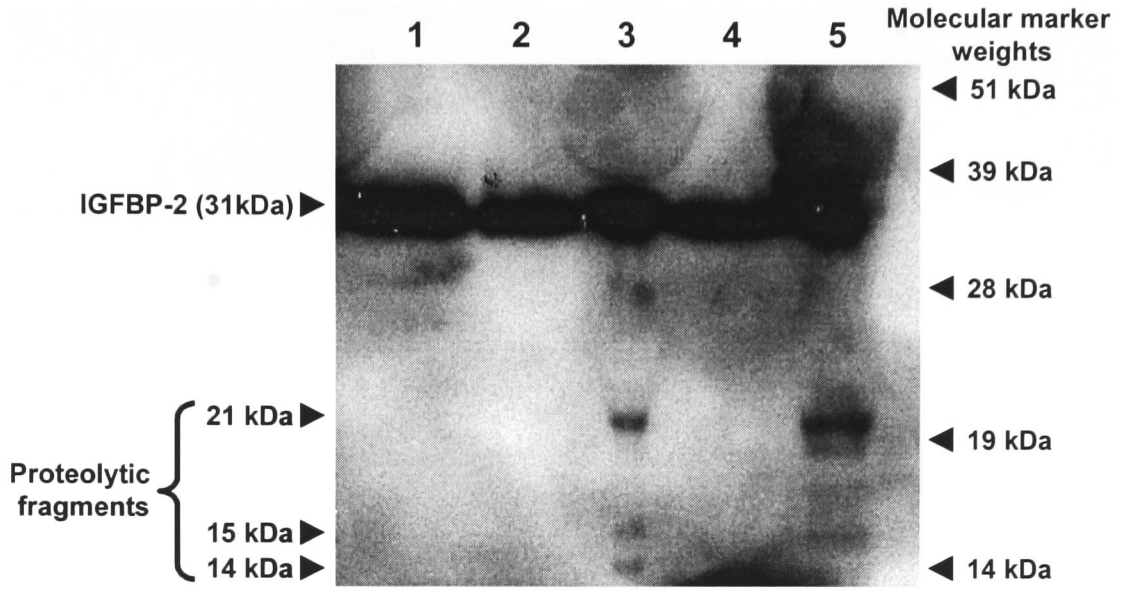


Figure 6.2

Representative immunoblot measuring IGFBP-2 proteolytic activity of bovine oocytes and granulosa cells

Lanes 1-3 and 5 are samples containing oocyte-cumulus complexes, denuded oocytes, mural granulosa cells and bovine follicular fluid respectively, which have been incubated with IGFBP-2. Lane 4 contains intact IGFBP-2.

Several breakdown products of IGFBP-2 – of approximately 21 kDa, 15 kDa and 14 kDa, as well as 21 kDa and 15-kDa – were identified from the incubation of mural granulosa cells and bovine follicular fluid (positive control) with IGFBP-2 respectively. No breakdown fragments were detected in the lane containing intact IGFBP-2 alone (negative control). In both the mural granulosa cell and follicular fluid samples the major breakdown product appeared to be at approximately 21 kDa. Incubation of IGFBP-2 with oocyte-cumulus complexes and denuded oocytes resulted in no proteolysis (Figure 6.2).

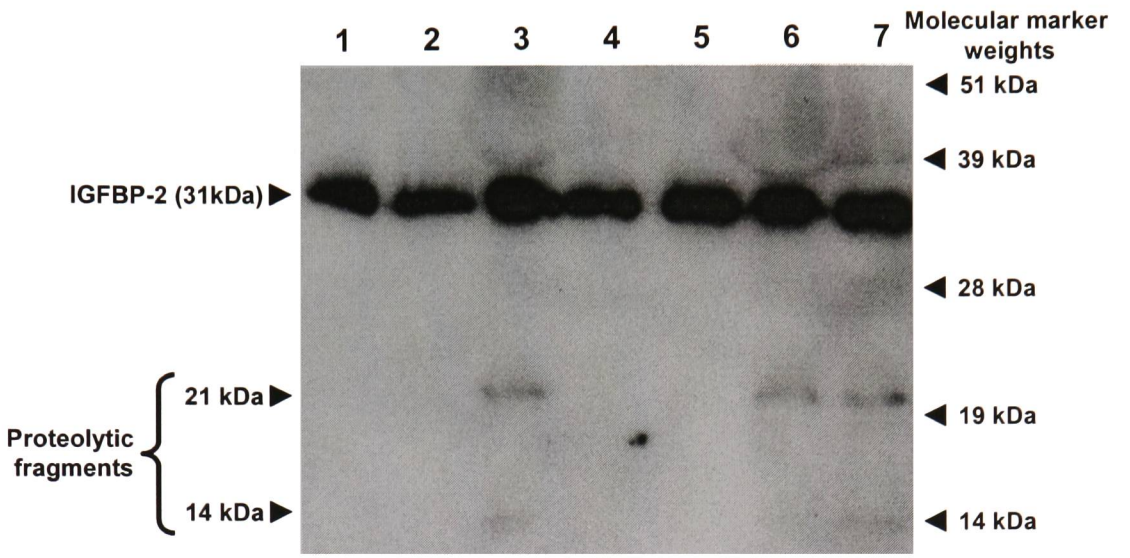


Figure 6.3

Representative immunoblot measuring IGFBP-2 proteolytic activity of bovine oocytes and granulosa cells in the presence of FSH or IGF-I

Lanes 1-3 are samples containing oocyte-cumulus complexes, denuded oocytes and mural granulosa cells respectively, which have been incubated with IGFBP-2 in the presence of FSH. Lanes 4-6 are samples containing oocyte-cumulus complexes, denuded oocytes and mural granulosa cells respectively, which have been incubated with IGFBP-2 in the presence of IGF-I. Lane 7 contains granulosa cells incubated with IGFBP-2.

Cleavage of IGFBP-2 was again observed after incubation of mural granulosa cells alone, and was also observed in the presence of FSH or IGF-I. Intact IGFBP-2 was found to migrate again at approximately 21 kDa and 14 kDa, with 21 kDa being the major breakdown product. The presence of FSH or IGF-I did not appear to inhibit or enhance proteolysis of IGFBP-2, brought about by the incubation of intact IGFBP-2 with mural granulosa cells. Oocyte-cumulus complexes and denuded oocytes incubated with intact IGFBP-2 in the presence of FSH or IGF-I failed to result in any proteolysis of IGFBP-2 (Figure 6.3).

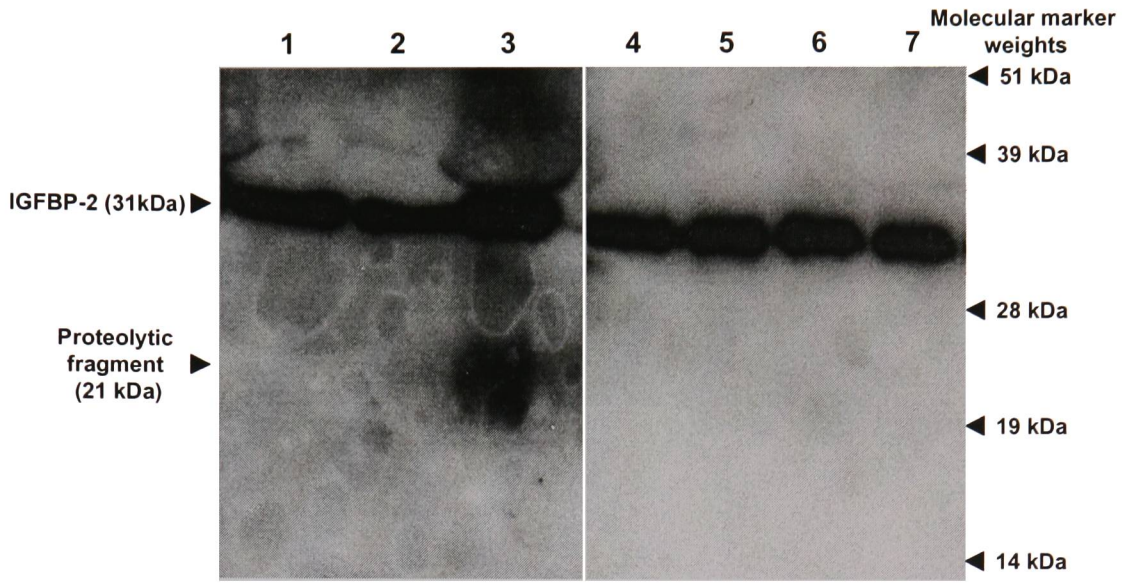


Figure 6.4

Representative immunoblot measuring IGFBP-2 proteolytic activity of bovine oocyte and granulosa cell concentrated conditioned media alone, or in the presence of IGF-I

Lanes 1-3 are samples containing oocyte-cumulus complex, denuded oocyte and mural granulosa cell concentrated conditioned media respectively, which have been incubated with IGFBP-2. Lanes 4 and 6 are denuded oocytes and denuded oocyte concentrated conditioned media respectively, which have been incubated with IGFBP-2 in the presence of IGF-I. Lanes 5 and 7 are samples containing oocyte-cumulus complexes and oocyte-cumulus complex concentrated conditioned media respectively, which have been incubated with IGFBP-2 in the presence of IGF-I.

To identify if bovine oocyte-cumulus complexes and denuded oocytes were producing proteases capable of cleaving IGFBP-2, but in very low concentrations, x10 concentrated samples of oocyte-cumulus complex (see section 6.2.2), denuded oocyte and mural granulosa cell conditioned media were incubated with IGFBP-2. Mural granulosa cell concentrated conditioned media was found to cleave IGFBP-2 with a breakdown product of approximately 21 kDa. However, intact IGFBP-2 was not found to be cleaved by a 20 hour incubation with oocyte-cumulus complex and denuded oocyte concentrated conditioned media; furthermore, the presence of IGF-I did not enhance proteolysis of IGFBP-2 (Figure 6.4).

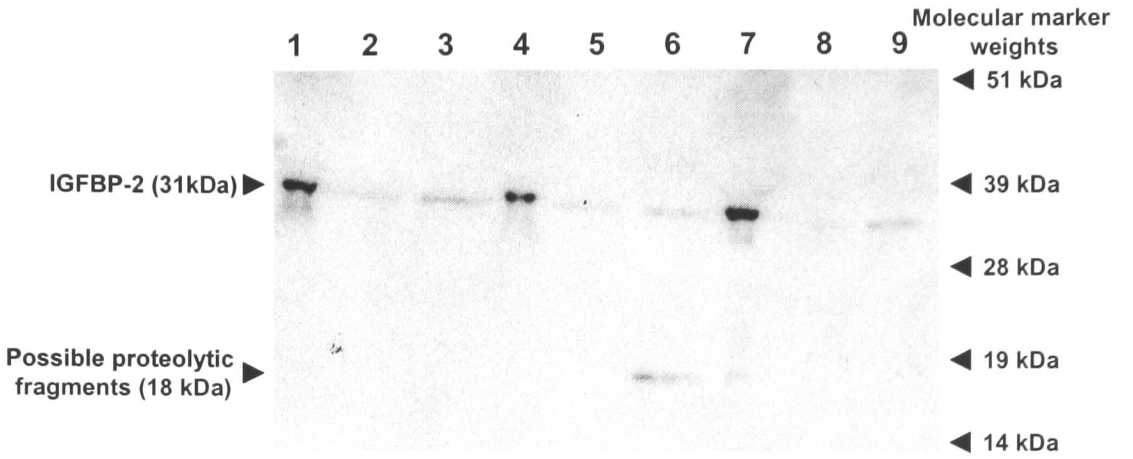


Figure 6.5

Representative ligand blot measuring IGFBP-2 proteolytic activity of bovine oocytes and granulosa cells

Lanes 1, 4 and 7 are samples containing mural granulosa cells incubated with intact IGFBP-2 alone or in the presence of IGF-I or FSH respectively. Lanes 2, 5 and 8 are samples containing denuded oocytes incubated with intact IGFBP-2 alone or in the presence of IGF-I or FSH respectively. Lanes 3, 6 and 9 are samples containing oocyte-cumulus complexes incubated with intact IGFBP-2 alone or in the presence of IGF-I or FSH respectively.

Western ligand blotting with biotinylated IGF-II revealed one band of 31 kDa, which corresponds to the intact IGFBP-2 added to the samples. One possible proteolytic fragment was observed in samples containing oocyte-cumulus complexes incubated in the presence of IGF-I and mural granulosa cells incubated in the presence of FSH. However, this result was very faint and not consistent, so this method does not appear to be ideal for detection of proteolytic fragments resulting from the degradation of IGFBP-2 (Figure 6.5).

6.4 Discussion

Evidence for proteolysis of IGFBP-2 has been detected previously in bovine (Monget *et al.* 2003; Spicer *et al.* 2001), ovine (Besnard *et al.* 1996b), porcine (Besnard *et al.* 1997; Monget *et al.* 2003) and equine (Bridges *et al.* 2002; Gerard *et al.* 2004) follicular fluid with the use of a combination of immunoblotting and western ligand blotting techniques. However, the exact location of the production of protease(s) capable of degrading IGFBP-2 remains unknown. The findings from the present study, consistent with previous studies, revealed that bovine follicular fluid was capable of cleaving IGFBP-2 (Spicer *et al.* 2001; Monget *et al.* 2003). Furthermore, bovine mural granulosa cells were shown to produce a protease, soluble in culture medium, which degraded exogenously added IGFBP-2, indicated by the production of low molecular weight fragments. The incubation of oocyte cumulus complexes and denuded oocytes with intact IGFBP-2 did not show any proteolysis of IGFBP-2, indicating that the protease(s) is mainly produced by mural granulosa cells. The addition of recombinant IGF-I or FSH had no effect in terms of enhancing or inhibiting the degradation of IGFBP-2 in any of the treatment groups.

Proteolytic activity for IGFBP-2 and -3 has been detected in culture medium conditioned with human theca and stroma (Mason *et al.* 1996). In the present study, the addition of FSH or IGF-I was not found to make IGFBP-2 more susceptible to cleavage by oocyte cumulus complexes or denuded oocytes. Furthermore, the level of proteolysis observed by mural granulosa cells after the addition of FSH or IGF-I did not appear to significantly alter when compared to the degree of proteolytic degradation caused by mural granulosa cells incubated in their absence. Unlike the current study, a variety of factors have previously been shown to have a role in modulating IGFBP proteolysis. In rats, treatment with pregnant mare serum gonadotrophin (PMSG) was shown to markedly increase the expression of the IGFBP-4 protease PAPP-A in mural granulosa cells, while FSH was found to stimulate PAPP-A expression in mural granulosa cells, as well as switching on expression of PAPP-A in cumulus granulosa cells. Interestingly, this FSH induced stimulation of expression of PAPP-A was inhibited in the presence of oocytes and the oocyte secreted factor BMP-15 (Matsui *et al.* 2004). FSH and IGF-I have both

been implicated in several species as playing a role in either enhancing or inhibiting IGFBP degradation (Adachi *et al.* 1995; Fielder *et al.* 1993; Gerard *et al.* 2004; Grimes and Hammond 1994; Iwashita *et al.* 1998; Liu *et al.* 1993; Mazerbourg *et al.* 2000; Monget *et al.* 2003). Studies using bovine and porcine preovulatory follicular fluid have shown that they were only capable of inducing a partial proteolytic degradation of exogenous IGFBP-2. However, this degradation was enhanced in the presence of excess IGF-II in a dose-dependent manner (Monget *et al.* 2003), suggesting that, like IGFBP-4 (Mazerbourg *et al.* 2000), IGFBP-2 might undergo a conformational change after binding to IGFs that make it more susceptible to degradation (Gockerman and Clemmons 1995). Moreover, the addition of LR3-IGF-I, which does not bind to IGFBPs, was less efficient than IGF-I or IGF-II at enhancing cleavage of IGFBP-2 (Monget *et al.* 2003).

Characterisation of specific IGFBP proteases will be beneficial to developing *in vitro* culture techniques, as it will allow us to tract follicle development and they may act as markers of follicle health. Identification of the protease PAPP-A in human ovaries by *in situ* hybridisation revealed that preantral and atretic antral follicles have very low levels of detection, while healthy antral to preovulatory follicles displayed an intense PAPP-A signal. This signal was restricted to the granulosa cells, and was also present in healthy corpora lutea (Hourvitz *et al.* 2000). By measuring IGFBP-4 protease activity with PAPP-A antibodies the secretion of PAPP-A has been detected from cultured human ovarian granulosa cells. However, PAPP-A IGFBP-4 protease activity is barely detectable in granulosa cell conditioned media until follicles are more than 9mm in diameter, which is coincident with dominant follicle selection in the human ovary. Additionally, secretion of PAPP-A was found from luteinising granulosa cells (Conover *et al.* 2001). IGFBP-2 and PAPP-A mRNA have been shown to be expressed in granulosa cells in sheep (Besnard *et al.* 1997; Mazerbourg *et al.* 2003) and cattle (Armstrong *et al.* 1998; Mazerbourg *et al.* 2001), with PAPP-A mRNA expression being highest in granulosa cells from fully differentiated follicles when compared to immature and atretic follicles (Mazerbourg *et al.* 2001). In addition, PAPP-A has been highlighted as a protease found in preovulatory bovine follicular fluid that is capable of cleaving IGFBP-2, with an increase in heparin-

binding fragments from degraded IGFBP-5 possibly blocking this PAPP-A cleavage of IGFBP-2 by direct interaction with PAPP-A rather than IGFBP-2 (Monget *et al.* 2003). Interestingly, even though PAPP-A expression is highest in fully differentiated bovine follicles (Mazerbourg *et al.* 2001), large dominant bovine follicles were found to have a lower IGFBP-2 proteolytic activity than subordinate follicles (Spicer *et al.* 2001). These results support the view that, other than the direct cleavage of IGFBP-2 by PAPP-A, various other proteases – such as kallikreins (Holland *et al.* 2001; Rehault *et al.* 2001) and/or other mechanisms – are involved in the loss of IGFBP-2 expression during bovine follicle development (Spicer 2004).

The intrafollicular cleavage of IGFBPs directly participates in the increase in bioavailability of IGFs that might further stimulate granulosa cell proliferation and steroidogenesis (Monniaux and Pisselet 1992). Proteolysis of human IGFBP-3 generates two types of fragments with different activities. One has a weak affinity for IGF-I and is a weak antagonist of IGF action; the other lacks affinity for IGFs but inhibits IGF-stimulated mitogenesis, thus acting via a mechanism independent of the IGFs (Lalou *et al.* 1996). It is, therefore, possible that IGFBP-2 proteolytic fragments have IGF-independent effects on follicular cells. Furthermore, it has recently been demonstrated that levels of one IGFBP can have direct effects on the level of other IGFBPs (Cwyfan-Hughes *et al.* 1997; Mazerbourg *et al.* 1999; 2000). The addition of exogenous IGFBP-2 to follicular fluid from ovine, bovine, porcine and equine preovulatory follicles was able to inhibit IGFBP-4 degradation, suggesting that alterations in intrafollicular IGFBP-2 levels, due to changes in mRNA expression and proteolytic degradation, are involved in the regulation of intrafollicular IGFBP-4 levels (Mazerbourg *et al.* 1999; 2000).

Overall, this data shows that in the developing bovine antral follicle, mural granulosa cells but not oocytes and cumulus granulosa cells have the ability to produce proteases capable of cleaving IGFBP-2. This data clearly demonstrates yet another mechanism by which IGF bioavailability can be regulated, and hence aids in modulating the specific actions of IGFs. Differences in the level of mRNA and protein of IGFBP-2 have been studied in Chapter 5; however, by studying the

production of proteases the actual bioavailability and processes involved in regulating the access of IGFs to the follicle can be revealed. Therefore, future research should focus on further characterisation and regulation of specific proteases and elucidation of the production and their spatial and temporal expression throughout ovarian follicle development.

The original hypothesis behind this study was that the oocyte was capable of producing proteases that would cleave IGFBP-2 at specific developmental stages, thereby increasing IGF bioavailability and hence IGF receptor mediated effects upon the developing follicle. This would provide a stage dependent putative mechanism whereby the oocyte can regulate its exposure – as well as the surrounding granulosa cells – to IGFs and thus control its own development as well as the early stages of granulosa cell differentiation and proliferation. Previous studies and work carried out for this thesis support this view, as IGF-I was not found to play an important role during primordial follicle initiation or early follicle development (see Chapter 3 results). The IGF receptor has been shown to be present from the preantral stage in bovine follicles (Armstrong *et al.* 2000; Armstrong *et al.* 2002) and has been shown to have a stimulatory role in follicle development (Gutierrez *et al.* 2000; Itoh *et al.* 2002). However, the finding that overexposure of preantral follicle oocytes to IGF-I had a negative effect on their health highlighted the importance of the correct regulation of IGF bioavailability. As the follicle develops to the antral stage it has been shown to be able to respond to the presence of IGF-I in a stage dependent manner. Granulosa cells were shown to proliferate and differentiate, IGF-I was found to improve oocyte health in the more mature antral follicles studied, and the expression of IGFBP-2 altered to either increase or decrease IGF bioavailability in response to the level of 'free' IGF available to the follicle (see Chapters 4 and 5). Although the oocyte was not found to produce protease(s) capable of cleaving IGFBP-2, there are limitations to this current study as the proteolytic activity of oocytes and granulosa cells from only antral follicles was investigated. Effects of the oocyte and granulosa cells can be stage dependent (Latham *et al.* 2004), so a future study into the production of proteases from oocytes and granulosa cells at specific developmental stages would give a fuller understanding of the mechanisms

regulating IGF bioavailability. The use of ^{125}I-labelled IGFBP-2 would account for problems associated with ligands not being able to bind sufficiently to the IGFBP fragments and possible cross-reactive and non-specific binding of the antibodies used. Additionally, if the exact protease is identified, then mRNA levels of this protease could be measured from the oocyte and/or granulosa cells collected from varying stages of follicular development.

mRNA for the protease PAPP-A has been shown to be expressed in the granulosa cells of bovine follicles, with expression being highest in granulosa cells from bovine fully differentiated follicles in comparison with immature and atretic follicles (Mazerbourg *et al.* 2001). Furthermore, PAPP-A has been highlighted as a protease found in preovulatory bovine follicular fluid that is capable of cleaving IGFBP-2 (Monget *et al.* 2003). It could therefore be hypothesised that the protease being produced by the granulosa cells is PAPP-A. However, to prove this the ability of a PAPP-A antibody to immunoneutralise the proteolysis of intact IGFBP-2 could be tested by supplementation of the PAPP-A antibody to the incubation culture medium. Moreover, the addition of protease inhibitors that inhibit different types of proteases – such as serine, cysteine or matrix metalloproteinases – would rule out the possible production of proteases other than PAPP-A.

CHAPTER SEVEN

General Discussion

7.1 Final conclusions

The development of *in vitro* systems to support the growth and development of follicles from domestic species would be advantageous as they provide an excellent model for investigating the long-term culture of immature follicles. The complete development of oocytes *in vitro* from the primordial follicle stage through to the production of live young has only ever been achieved in rodents (Eppig and O'Brien 1996; O'Brien *et al.* 2003). To date, there has been little success in applying these techniques to humans and domestic animals that have follicles that require a long growth period. In recent years it has become clear that local control of follicular development by growth factors plays a crucial role in the regulation of follicle development and oocyte maturation. The insulin-like growth factor system is one of the local control families that have been found to play a key role in regulating follicular growth, and a fuller understanding of the mechanisms involved in the regulation of IGF could improve *in vitro* culture systems.

The aims of this thesis were to (1) investigate the effects of IGF-I on early bovine oocyte and somatic cell development and health using a defined serum-free culture system, (2) analyse temporal and spatial differences in IGFBPs expression at different stages of follicle development, as a mechanism to regulate IGF bioavailability and (3) identify the proteolytic activity of different bovine follicular compartments. The research carried out in this study has revealed that IGF-I can have a direct action on the developing follicle, and that the follicle is capable of regulating its own exposure to IGFs by modulating IGFBP expression. Furthermore, these effects are both dose and stage dependent. In addition, the secretion of proteases capable of degrading IGFBP-2 was detected in granulosa cell conditioned medium but not oocyte conditioned medium.

The gradual initiation of growth of ovarian follicles from the primordial pool is a process that is poorly understood. In Chapter 3 the effect of IGF-I on primordial initiation and early follicle growth and oocyte health, in the presence or absence of androgen, was investigated. Previous studies have shown androgen receptors to be detected in bovine follicles (Hampton *et al.* 2004), and work in this thesis confirmed

the presence of androgen receptor immunoreactivity in granulosa cells and oocytes of preantral and antral bovine follicles. In addition, androgen has been shown in previous studies on primate ovaries to promote primordial follicle initiation and increase IGF-I and IGF-I receptors gene expression (Vendola *et al.* 1999a; 1999b). Taken together, these results imply that androgens may play a role in early follicle growth and that the actions of IGF-I through its receptor may be influenced by the presence of androgens. The current data, in agreement with previous studies (Derrar *et al.* 2000; Kezele *et al.* 2002b), found no effects of IGF-I on primordial follicle initiation or activated follicle growth. Supplementation of the culture medium with androstenedione, either on its own or in combination with IGF-I, also failed to affect follicle activation and growth. IGF-I that was not regulated by IGFBPs was found to have a negative effect on the oocyte health of follicles cultured for six days. This suggests that during the early stages of follicle development, as seen in follicles at the preantral stages of development (McCaffery *et al.* 2000), the regulation of the bioavailability of IGF is crucial to maintain the health of the oocyte. This result was not altered by the supplementation of the culture medium with androstenedione, suggesting that the actions of IGF-I through its receptor are not influenced, at this stage in bovine follicle development, by the presence of androgens. In summary, this data shows that in the developing bovine follicle IGF-I was not found to play an important role during primordial follicle initiation or early follicle development. However, as this and other studies have shown the culture of cortical strips causes wholesale activation of follicles (Braw-Tal and Yossefi 1997; Derrar et al. 2000; Wandji et al. 1996, see Chapter 3 results). This activation does not appear to be normal and is probably as a result of the overstimulation by the inappropriate culture conditions. Due to this fact, the use of the CAM culture system, which has been shown not to cause mass initiation of primordial follicles (Cushman *et al.* 2002), may prove to be a more useful technique in identifying factors that influence primordial follicle activation and early follicle growth.

The precise role of the IGF system in the regulation of bovine oocyte and follicle development throughout follicle growth is now becoming more fully understood (Derrar *et al.* 2000; Itoh *et al.* 2002; Lorenzo *et al.* 1994; McCaffery *et al.* 2000).

IGF-I has been shown to be essential for follicle development past the late preantral stage (Baker *et al.* 1996), to stimulate both proliferation and differentiation of somatic cells (Spicer *et al.* 1993; Stewart *et al.* 1995), to act as a follicle survival factor (Louhio *et al.* 2000), to stimulate follicle development (Gutierrez *et al.* 2000; Itoh *et al.* 2002) and to enhance nuclear maturation of oocytes (Lorenzo *et al.* 1994). However, the regulation of IGF bioavailability appears to be as important as its presence, as previous culture of bovine preantral follicles has shown that while IGF-I had a positive effect on follicle growth, a negative effect was observed on oocyte size and granulosa differentiation (McCaffery *et al.* 2000). In the present study (Chapter 4), human recombinant IGF-I, when not regulated (1μg/ml), was found to significantly stimulate follicle proliferation in the early stages of antral development, and differentiation at all developmental stages investigated, as exhibited by oestradiol production. Furthermore, these effects were found to occur in a dose and stage dependent manner. Interestingly, LR3 IGF-I, even though it is not regulated by IGFBPs, did not stimulate follicle differentiation or proliferation, indicating that the high dose of recombinant IGF-I may have been acting via the insulin receptors as well as the IGF receptors. Moreover, in the most mature antral follicles studied, oocyte health was found to improve by the presence of human recombinant IGF-I. The results emphasise the continual changes in the requirements of the developing follicle and the mechanisms regulating the IGF system throughout follicular development. Follicular growth is regulated by the interaction of many different factors. One of the main roles of IGFs is to work in synergy with FSH to regulate follicle development, hence, future studies investigating the interaction of IGFs and FSH during antral growth would provide a more comprehensive understanding of how these factor may work together *in vivo*. Furthermore, IGF-II has been shown to be the principal intrafollicular IGF ligand in cattle (Armstrong *et al.* 2000), hence, investigation into it actions and identification of expression patterns of the type II IGF receptor would be beneficial.

The biological actions of IGF depend in part on the ability of specific proteases to break down the IGF/IGFBP complex and/or the level of expression of the IGFBPs (Giudice 1992). In Chapter 5 it was shown that IGF-I could exert an influence on the

regulation of its own bioavailability, by influencing the expression of IGFBP-2 in the oocyte and granulosa cells. The results suggest that IGF-I may regulate the expression of IGFBP-2 through a negative feedback mechanism, which again is dose and stage dependent. In the present study there was found to be a significant increase in oestradiol production in the follicles cultured in the high dose of IGF-I (see Chapter 4 results), which also exhibited a high level of IGFBP-2 expression. Oestradiol in the presence of insulin has been shown previously to increase IGFBP-2 mRNA expression in granulosa cells of antral follicles (1-5mm in diameter) (Voge *et al.* 2004). Hence further work on the regulation of IGFBPs by growth factors working alone or in synergy with gonadotrophins or steroids will give a further understanding of the relative bioavailability of IGF present at different follicular development stages.

In addition to the level of expression of IGFBPs, the release of specific IGFBP proteases at specific stages of development is yet another mechanism that plays an important role in modulating IGF bioavailability (Spicer *et al.* 2001; 2004). Chapter 6 explored the proteolytic activity of oocytes and granulosa cells from large antral follicles by incubating them in the presence of intact IGFBP-2 and then identifying the presence of proteolytic fragments brought about by the cleavage of IGFBP-2. Mural granulosa cells but not oocytes or cumulus granulosa cells were found to secrete proteases capable of degrading IGFBP-2. Cleavage of IGFBPs has been shown to be enhanced or inhibited by the presence of IGF-I (Fielder *et al.* 1993; Grimes and Hammond 1994) and/or FSH (Adachi *et al.* 1995; Liu *et al.* 1993; Fielder *et al.* 1993). However, in the present study, the addition of FSH or IGF-I was not found to make IGFBP-2 more susceptible to cleavage by incubation with oocytes or granulosa cells when compared to the degree of proteolytic degradation caused in their absence. The hypothesis that the oocyte and/or granulosa cells were capable of producing proteases that would cleave IGFBP-2 at specific developmental stages, thereby increasing IGF bioavailability and hence IGF receptor mediated effects upon the developing follicle; provides a stage dependent putative mechanism whereby the oocyte and/or granulosa cells can regulate their own exposure to IGFs and thus control their own development as well as the early stages of granulosa cell

differentiation and proliferation. Oocyte were not found to produce a protease(s) capable of cleaving IGFBP-2 which does not seems surprising as the production of a protease from the oocyte may be potentially very damaging if it were to be activated at an inappropriate time. The concentration of IGFBP-2 in bovine follicular fluid is much higher than that of IGFBP-4 and -5 (Spicer and Echternkamp 1995), and furthermore IGFBP-2 has been shown to be bound up in granulosa cells, hence, a protease capable of cleaving IGFBP-2 would have to be strictly regulated to ensure the release and hence increase in IGFs bioavailability occurs in a regulated fashion. It seems much more likely that the oocyte may stimulate the granulosa cells to start producing a protease capable of cleaving IGFBP-2. Effects of the oocyte and granulosa cells can be stage dependent (Latham et al. 2004), so a future study into the production of proteases from oocytes and granulosa cells at specific developmental stages would give a fuller understanding of the mechanisms regulating IGF bioavailability. The addition of protease inhibitors that inhibit different types of proteases – such as serine, cysteine or matrix metalloproteinases – or the addition of specific protease antibodies would allow the exact protease(s) to be identified. Additionally, if the exact protease is identified, then mRNA levels of this protease could be measured from the oocyte and/or granulosa cells collected from varying stages of follicular development. Further work identifying the developmental stages when specific proteases that degrade IGFBPs are being produced would allow us to monitor follicular development during long-term culture, as they may act as markers of follicular health and thus follicle viability.

Overall, these experiments highlight the importance of the IGF system in the early stages of oocyte and follicle growth, and emphasise the need for regulatory mechanisms, such as the IGFBPs, to be maintained during *in vitro* follicle culture. The results presented here draw attention to the need for future *in vitro* work to focus on investigations into the regulation of ovarian follicle development, as these are essential to our understanding of how the oocyte gains full developmental competence. It is still unclear how paracrine factors co-ordinate follicle and oocyte development, especially during the early stages of growth. Locally produced growth factors are usually secreted via the constitutive secretary pathway and stored in

extracellular depots where they are unable to interact with their receptors. Therefore, the complex mechanisms regulating the release or activation of growth factors that are associated with the ECM, attached to cell surfaces, or sequestered by carrier/binding proteins, is vital to elucidate the regulation of the bioavailability of these important factors during follicle development. Furthermore, the identification of good developmental markers, such as the production of proteases at specific developmental stages, is needed to enable advancement in the development of an *in vitro* culture system that will accelerate follicle development without causing precocious differentiation to occur.

7.2 Concluding remarks

Bovine and primate ovaries have many similarities that make the cow an excellent model for humans. Bovine ovaries are also an important model in themselves because of their agricultural importance. The ultimate goal of studies on follicle growth *in vitro* is to develop conditions that will sustain follicular development to the stage where the oocyte is capable of meiotic maturation, fertilisation and normal development.

The use of serum-free cultures as a way to improve our understanding of how growth factor systems are regulated during follicle development has provided a wealth of knowledge in recent years. The aim of this thesis was to use these culture techniques to provide insights into how the complex IGF system is regulated throughout follicular growth. This research has highlighted the stage and dose dependent actions of IGF-I on both follicle proliferation and differentiation, as well as oocyte health. In addition, the regulation of IGF bioavailability by IGFBPs has been shown to be governed, in part firstly, by the level of expression that can be modulated by the presence of IGFs and follicle developmental stage; and secondly, the production of specific IGFBP proteases by specific cell types. Further investigation into how the IGF system interacts with gonadotrophins and other local growth factors will give a broader understanding of the multifactorial regulatory mechanisms governing follicle development.

Culture systems are of great importance as they not only provide an environment for *in vitro* growth of follicles but are also an invaluable tool for further research into processes governing follicular development. Only with continued research into the precise regulatory events of growth factor systems, such as the IGF system, will a fuller understanding of the complex processes involved in normal follicle development and oocyte maturation be gained.

CHAPTER EIGHT

References

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